University of Alberta

Regulation of CTL Generation by IFN-γ in Alloimmune Responses in the Mouse

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Immunology

Department of Medical Microbiology and Immunology

Edmonton, Alberta

Fall, 2005

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Dedicated to:

Isaac Aurelio Hidalgo Peralta

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ABSTRACT

IFN-γ is an effector cytokine produced by T cells and NK cells during allograft rejection. CTL constitute a major portion of the infiltrate in rejecting allografts, produce IFN-γ upon TCR engagement, and respond to IFN-γ. We sought to understand the mechanisms by which IFN-γ regulates CTL responses, *in vitro* and *in vivo*.

We examined the effect of IFN-γ on CTL generation *in vitro* in MLC and *in vivo*. IFN-γ decreased the percent of CTL generated resulting in decreased lytic activity. This was mediated by decreased IL-2 due to decreased CD4 T cell proliferation. The effects on CD4 T cells were mediated through IRF-1 since IRF-1 -/- responders also displayed increased proliferation and IL-2 production. A similar effect was observed *in vivo*. IFN-γR1 -/- mice generated more CTL and IL-2 in response to allogeneic tumor challenge, and CTL generation was IL-2 dependent. Thus IL-2 increases CTL generation and IFN-γ negatively regulates CTL generation by acting on CD4 T cells to decrease IL-2 production forming a negative feedback loop.

We assessed the effects of IFN-γ on the cellular infiltrate and allogeneic tumor cell clearance in response to intraperitoneal challenge. IFN-γR1 -/- hosts contained increased numbers of macrophages and granulocytes in the infiltrate compared to wild-type mice. Despite increased CTL generation in SLOs the number of CTL recruited to the inflamed site was not increased. Furthermore, a greater number of allogeneic tumor cells remained in IFN-γR1 -/- hosts compared to wild-type. Thus IFN-γ greatly affects

the cell types, and numbers that are recruited to the site of rejection and plays a role in the control of allogeneic tumor grafts.

These studies demonstrate a mechanism where IFN-γ negatively regulates CTL generation *in vitro* and *in vivo*. Our results contribute to understanding CTL generation and allude to unique effects of IFN-γ on specific T cell subtypes. We have shown independent homeostatic control of lymphoid organs compared to the inflammatory compartment. Furthermore, we have shown that IFN-γ promotes the destruction of allogeneic tumor cells, despite limiting CTL generation. These are important findings in understanding the mechanisms of allograft rejection and the associated role of IFN-γ.

Acknowledgement

I have many people to thank for their support during the time in which I developed as a person and a scientist.

I would first like to acknowledge my family: My mother Maria Lucero, my brother Diego Lucero, and my uncle Cesar Hidalgo. Thank you for the encouragement throughout my post-secondary studies and for making the visits home such a reward. To my girlfriend Kathryn Hagedorn: thank you for being a bright light during the dark times of this degree and my life, I could not have finished this without you.

To Phil Halloran: I am indebted to you for many things. For teaching me how to think like a scientist and not letting me forget about the 'big picture' which is so often overlooked in science. For developing and maintaining a lab with such a strong drive for results that manages to maintain a great camaraderie. For these and many other things I consider you a role model and a friend.

To the Halloran lab over my time here: Joan Urmson, Vido Ramassar, Pam Publicover, Lina Kung, Tasha Sims, Anette Melk, and Kara Allanach. Thank you for being my friends and colleagues. It is you who made the lab an enjoyable and safe place to work.

This work was supported by grants from the Canadian Institute for Health Research, The Kidney Foundation of Canada, The Muttart Endowment, The Royal Canadian Legion, Fujisawa Canada, Novartis Canada, and Roche Canada.

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CHAPTER 1

BACKGROUND

1.1 INTRODUCTION

Allograft rejection invokes a powerful immune response initiated by the activation of alloreactive T cell clones. Alloreactive T cells undergo clonal expansion in secondary lymphoid organs (SLOs) to generate a large number of effector CD4 and CD8 T cells. CD4 T cells help the generation of the immune response through the production of cytokines and expression of costimulatory molecules critical for CD8 T cell expansion and B cell antibody production. Effector CD4 T cells enter allografts where they influence every aspect of the immune response through the production of effector cytokines. Effector CD8 T cells, or cytotoxic T lymphocytes (CTL), make up the majority of the T cell infiltrate in rejecting allografts (1). CTL mediate their effector function mainly through contact-dependent, target cell lysis but are also potent producers of effector cytokines. Thus CD4 T cells are critical in the initiation of alloimmune responses and both CD4 and CD8 T cells can alter the immune response at the inflamed site through the production of effector cytokines.

IFN-γ is the prototypical effector cytokine able to affect both the generation of the immune response in SLOs as well as the development of the immune response in inflamed tissue. Both CD4 and CD8 effector T cells, and natural killer (NK) cells produce IFN-γ in large quantities. NK cells play a role in the initiation of immune responses being the primary source of IFN-γ in SLOs early in the response (2). At

affected sites in the periphery, IFN-γ acts on both hematopoeitic and non-hematopoeitic cells to alter the leukocytic infiltrate and enhance clearance of intracellular pathogens. Due to its many effects at all stages of the immune response, the levels of IFN-γ in SLOs and inflamed tissue affect both the strength of the response generated and the recruitment of inflammatory cells.

1.2 OVERVIEW OF IFN-γ

The interferons (IFNs) are widely expressed cytokines that mediate antiviral, anti-proliferative, and immunoregulatory effects. IFNs are primarily divided into two families of related cytokines: Type I IFNs and Type II IFN.

Type I IFNs include IFN-α, IFN-β, IFN-κ, IFN-ω all having highly homologous structures. The receptor for Type I IFNs is widely expressed, allowing for their potent antiviral activity on various cell types. Production of Type I IFNs is not restricted, and can be produced by nearly any cell type upon viral infection. The ability of plasmacytoid dendritic cells (DCs) to produce Type I IFNs has generated renewed interest in their potential immunoregulatory effects. Immunoregulatory effects of Type I IFNs have previously been described (3).

In contrast to Type I IFNs there is only one Type II IFN: IFN-γ. IFN-γ is a pleiotropic cytokine that plays key roles in the modulation of many aspects of an immune response. Regulation of gene expression by IFN-γ is mediated through its widely

expressed receptor that bears little resemblance to the Type I IFN receptor. As a potent immunomodulator, IFN-γ exerts effects on all immune cells, but most non-marrow derived cells also respond to IFN-γ. Included in its list of effects IFN-γ enhances detection of pathogens through its effects on antigen presentation, inhibits viral growth and cell proliferation, and facilitates the recruitment of effector T cells to areas of infection.

Because of its strong immunomodulatory effects, IFN-γ expression is restricted to certain cells of the immune system. The major producers of IFN-γ are CD4 and CD8 T cells, B cells, and NK cells (4). In T cells, IFN-γ gene expression is induced following T cell receptor (TCR) stimulation, and is followed by IFN-γ protein secretion. CD8 T cells maintain their production of IFN-γ while their TCR is engaged with antigen in the context of MHC class I, and production is stopped upon disengagement (5). Macrophages and DCs can be induced to express IFN-γ under extreme circumstances (6) but the contribution of this during an immune response is unclear.

IFN-γ production can be affected by cytokines produced by antigen presenting cells (APCs). IL-18, the IL-12 family, transforming growth factor (TGF)-β and IL-10 are central cytokines in the regulation of IFN-γ. IL-12 and IL-18 induce IFN-γ gene expression in T cells and NK cells (7), with IL-1β being a more potent inducer (8). In contrast, TGF-β and IL-10 reduce IFN-γ production by T cells directly, and by inhibiting IL-12 production from APCs, respectively (9).

1.3 REGULATION OF IFN-γ EXPRESSION

Like other genes, IFN-γ gene expression is regulated by transcription factors that interact with specific sites on the IFN-γ promoter. The structure of the IFN-γ gene consists of four exons and three introns of sizes that are highly conserved amongst species. Located on chromosome 12 in the human, or chromosome 10 in the mouse, expression of the gene leads to the production of a dimeric protein. The IFN-γ protein is a head to tail dimer composed mostly of α-helices with an overall compact and globular structure (10). Although the folding topology of each IFN-γ monomer is similar to that of IFN-β, the dimeric structure of IFN-γ is unique amongst interferons. Being a dimer, IFN-γ binds to two IFN-γR1 chains which then recruit IFN-γR2 chains to initiate a signaling cascade leading to initiation of transcription. The spectrum of genes induced by IFN-γ varies depending on the cell type and by the presence of other cytokines which may synergize or antagonize its effects.

a) The IFN-γ gene. Within the IFN-γ gene, the promoter is the most highly conserved portion. Sites that resemble the nuclear factor-activated T cell (NF-AT) binding sites (11) in the IL-2 promoter are found in the promoter and in the first intron. The role of these sites, and of NF-AT proteins in the regulation of IFN-γ production by activated T cells is not clear (12). Enhancer elements are present in the IFN-γ promoter as well as in the introns, and these interact with NF-κB proteins in a calcineurin-dependent manner when cells are activated (11) (Figure 1 a). As other mammalian gene promoters, the IFN-γ promoter contains conserved CpG sites that can be methylated leading to inhibition of gene expression. Thus, cells that express IFN-γ will normally

have an unmethylated promoter while those which express little or no IFN-γ will have a methylated promoter (13). Sites specific for signal transducers and activators of transcription (STAT) proteins, YY1, CREB, and AP-1 are also present in the IFN-γ promoter and are important in the regulation of IFN-γ expression (14).

Cell-specific expression of IFN-γ also requires expression of the transcription factor T-bet (T box expressed in T cells) (15). The IFN-γ gene locus contains three T box sites; two are upstream of the transcription initiation site and one in the third intron (15). IFN-γ can augment its own expression (16) perhaps through a positive feedback loop involving the induction of T-bet expression (17). However, IFN-γ -/- mice upregulate MHC class I expression in response to recombinant IFN-γ comparable to wild-type mice (18), thus it is uncertain how important self-augmentation by IFN-γ is in *in vivo* responses.

b) IFN-γ production elicited by non-T cells. Potent IFN-γ responses can be elicited by activating non-T cells. Both T cell dependent stimuli and T cell independent stimuli (oxazalone, lipopolysaccaride (LPS)) induce IFN-γ-dependent systemic MHC expression (19;20). T cell deficient mice make abundant IFN-γ, presumably of NK cell origin. Thus bacterial LPS induces IFN-γ production at least as strongly in T cell deficient nude and SCID mice as in wild-type mice (20). It is possible that NK cells are the main source of IFN-γ production in response to LPS (21), although B cells also respond strongly to LPS and can produce IFN-γ (22).

c) *Effects of IFN-γ production in the basal state.* IFN-γ production is evident even in the basal state. It is unclear which cells produce IFN-γ in the basal state but T cells do not seem to be required. NK cells may be responsible for basal IFN-γ production since basal MHC expression is not reduced in T cell-deficient nude mice (20). Whether other cytokines such as TNF also participate in induction of basal level of MHC in vascular endothelium is unclear (23). However, germ-free mice do not have reduced basal MHC expression, arguing against LPS from commensal bacteria as the trigger for basal IFN-γ production. Thus the stimulus for basal production of IFN-γ is unknown, but the source is likely not T cells and the stimulus is probably not LPS.

1.4 THE IFN-γ RECEPTOR AND IFN-γ SIGNALING

a) *The IFN-γ receptor.* Unlike the restricted production of IFN-γ, the IFN-γ receptor is ubiquitously expressed on the majority of cells. The receptor is composed of two R1 (α) and two R2 (β) chains each containing a single transmembrane domain (24). Both R1 and R2 belong to the Type II cytokine receptor family but play different roles in the interactions with IFN-γ. IFN-γR1 is primarily responsible for the binding of IFN-γ whereas IFN-γR2 is thought to initiate the signaling cascade from the receptor (25) however, expression of both chains is necessary for proper responses to IFN-γ (26;27). T cells generally express the R1 chain constitutively on their surface while surface expression of the R2 chain is variable. IFN-γR2 expression is induced by activation of

human peripheral blood T cells (28) and downregulated by IFN-γ stimulation in polarized T cells (29). However, most non-hematopoeitic cells express both chains of the IFN-γR constitutively thus are always responsive to IFN-γ. Unlike other cytokine receptors, IFN-γ receptors bind their ligand in a species-specific manner possibly as a result of only modest cDNA and amino acid homology for both the receptor and ligand between species despite conserved overall structure (4).

b) IFN-γ signal transduction. Signaling from the IFN-γR is mediated through the Janus kinase (JAK)-STAT pathway. JAK-STAT signaling is used by most cytokine receptors but IFN-γ specifically signals through JAK-1 and JAK-2 (30) which phosphorylate STAT1α, and possibly STAT3 (31). JAK-1 and JAK-2 are tyrosine kinases that constitutively associate with the IFN-γR1 and R2 chains, respectively, and STAT proteins are transcription factors that dimerize in response to cytokine receptor signaling (32). In unstimulated cells these JAK proteins are normally present in an inactive state. Binding of IFN-γ brings IFN-γR1 and IFN-γR2 chains within close proximity. Within this complex, JAK-1 and JAK-2 are able to transactivate each other and phosphorylate the IFN-γR1 chains at specific tyrosine residues (33). STAT1α recognizes the phosphorylated forms of the IFN-γ receptor chains through Src-homology (SH)2 domains, allowing recruitment and binding. Following recruitment, STAT1α is phosphorylated by JAK-1 and -2 at tyrosine 701, dissociates from the receptor and forms homodimers. STAT1 homodimers enter the nucleus and begin transcription of IFN-γ inducible genes (34) (Figure 1 b).

c) Transcription factors that mediate IFN-γ induction of gene transcription. IFN-γ regulates gene expression through direct and indirect effects on transcription factors. The transcription factors regulated by IFN-γ can be divided into three groups: first, second, and third generation transcription factors.

First generation factors are preexisting cytosolic factors. STAT1α is the critical first generation transcription factor for IFN-γ mediated changes. Binding of IFN-γ to its receptor leads to formation of STAT1 homodimers that localize to the nucleus and bind to gamma activated sites (GAS) present in the promoter region of many IFN-inducible genes (35). GAS sites include the consensus sequence TTA/CCnnnAA. The phenotype of STAT1α -/- mice is very similar to that of IFN-γ -/- mice, indicating that the signaling from the IFN-γR is specific and unique (36). Examples of genes immediately activated by IFN-γ include second generation transcription factors such as the interferon regulatory factor (IRF) family members (37).

Some members of the IRF family are IFN-inducible and are considered second generation factors, including IRF-1 (35), IRF-2 (37), interferon stimulated gene factor 3 γ (ISGF3γ/p48/IRF-9) (38), and interferon consensus sequence binding protein (ICSBP/IRF-8) (39). Two other members of this family; IRF-5 and IRF-7 are IFN-inducible, however, little is known of IRF-5, and IRF-7 appears to be strictly involved with Type I IFNs (40).

Consensus sequences for distinct transcription factors of the IRF family members are found on the promoters of many IFN-γ regulated genes. All IRF family members

show moderate homology in their DNA-binding domains yet differ in their transcriptional activation domains, suggesting individual functions for different IRF family members. Regulation of IFN-γ responses may also arise from the ability of IRF factors to form homo- or heterodimers to either enhance or suppress responses (41). Homology at the DNA-binding domain gives IRF family members the ability to recognize related elements including the interferon stimulated response element (ISRE), IRF binding element (IRF-E) and interferon consensus sequence (ICS). ISREs, IRF-E, and ICS are very similar and all contain the consensus sequence composed of repeats of GAAAnn (42). The ability of IRF family transcription factors to bind various consensus sequences is exemplified by the interaction of IRF-1 with different IFN-γ-inducible genes. For example, in response to IFN-γ stimulation, IRF-1 induces expression of MHC class I via an ICS, nitric oxide synthase (NOS)-2 via ISREs, and the class II transactivator (CIITA) promoter IV via IRF-E (43;44).

Despite the many similarities that exist between different members of the IRF family, differences exist in function, tissue expression, association with other proteins, and recognition of consensus sequences. IRF-1 and IRF-2 have similar DNA binding specificities but can function as a transcriptional activator or repressor of IRF-1 controlled sites, respectively (45;46). ISGF3 is composed of IRF-9 in association with STAT1 and STAT2 proteins and binds to ISREs to induce transcription of various genes. IRF-8 is similar to IRF-9 but is only expressed in lymphoid cells and binds to ISRE elements to negatively regulate genes that are activated by IRF-1 and IRF-9 (42;47). Differences in function displayed by IRF members are also reflected in their regulation of CD4 T cell differentiation to T helper (Th) 1 or Th2 phenotypes (48).

Third generation transcription factors are induced by second generation factors, often in synergy with first generation factors. These factors can regulate genes that may not appear to be IFN-γ inducible due to their lack of GAS or IRF-1 consensus sequences. A prototypical third generation factor is CIITA. CIITA is directly regulated by IFN-γ through STAT1α interaction with the GAS as well as through IRF-1 interaction with the IRF-E site that are present on promoter IV of the CIITA promoter (49). IFN-γ induced expression of CIITA allows for increased MHC class II expression.

1.5 IFN-γ EFFECTS

Due to the nearly ubiquitous distribution of the IFN-γR, IFN-γ production has effects on the majority of cells including both marrow and non-marrow derived cells. IFN-γ was originally identified for its antiviral activity but it mediates many other biological effects including regulation of MHC class I and class II expression, regulation of NOS2 and nitric oxide (NO), anti-proliferative effects, and regulation of leukocyte-endothelium interactions through effects on adhesion molecules as well as chemokines. Furthermore, within the immune system IFN-γ is a key regulator of innate and adaptive immunity.

a) Antiviral effects. Antiviral activity was the first characteristic that identified IFNs (50). All IFNs are able to make cells more resistant to viral infection, although the antiviral activity of IFN-γ is not as potent as that of Type I IFNs (4). The direct antiviral

effects of IFNs, are primarily due to the induction of three enzymes: double-stranded (ds) RNA activated protein kinase (PKR), dsRNA specific adenosine deaminase (dsRAD), and 2'-5' oligoadenylate synthetase (OAS). PKR and dsRAD are both activated by dsRNA and mediate the inhibition of protein synthesis and degradation of viral dsRNA, respectively (51;52). dsRNA also activates OAS which degrades viral and cellular ssRNA ultimately leading to inhibition of protein synthesis as well as viral replication (52). Through the regulation of these three enzymes, IFN-γ and Type I IFNs play an important role in aiding antiviral responses via direct effects on infected and uninfected cells. It is possible that the antiviral effects of IFN-γ may also mediate some of its anti-proliferative effects.

b) Regulation of MHC class I and class II. Expression of MHC class I genes is mainly regulated at the level of transcription. Induction of MHC class I transcription by IFN-γ is mediated through NF-κB and IRF-1 binding to the cAMP-response element (CRE) and ICS, respectively, in the MHC class I heavy chain promoter (53) (Figure 1 c). MHC class I expression requires IRF-1, as demonstrated by the lack of MHC class I in IRF-1 -/- mice (54). TNF can further enhance class I expression by acting through NF-κB, which synergizes with IRF-1, by binding to NF-κB sites found in the Enhancer A region of the class I promoter (55). Many other genes involved in MHC class I antigen presentation are also IFN-γ inducible in an IRF-1 dependent manner including β_2m, TAP1 and 2, LMP 2 and 7, as well as some ER chaperone proteins (56). The similar responses of these genes to IFN-γ results from similarities in their promoter regions. For

example, β_2m transcription levels parallel those of MHC class I due to the presence of an ICS and an NF-κB site in both promoters.

Many of the key genes of the MHC class II antigen-presentation pathway are also regulated by IFN-γ. Various transcription factors that directly bind to the MHC class II promoter are required but not sufficient for MHC class II expression. The transactivator CIITA is obligatory for constitutive and induced MHC class II expression, although it does not itself bind DNA (57). Four promoters in the human and three in the mouse regulate CIITA expression, determine cell-type specificity, and induction of MHC class II gene expression. Promoter I is used by DC, promoter III by B cells, and promoter IV (PIV) mediates IFN-γ-induced expression (58). Thus some MHC class II expression is IFN-γ independent but in the context of inflammation its expression is primarily driven by IFN-γ (59).

c) Anti-proliferative effects. The anti-proliferative effects of IFN-γ are mainly mediated through IRF-1 (31). These effects are not absolute however, and can at times be overcome with high concentrations of growth factors in certain hematopoietic cells (60). The overall ability of IFN-γ to inhibit cell growth likely involves the activation of various pathways that block cell cycle progression at various stages.

The cell cycle has a number of checkpoints some of which are regulated through cyclin-dependent kinases (CDK)-cyclin complexes (61). Families of CDK inhibitors such as $p16^{INK4}$, $p21^{WAF1/CIP1}$, and $p27^{Kip1}$ inhibit cell proliferation by binding to and inhibiting various cyclin-CDK complexes as well as components of the DNA replication

machinery (62-64). For a cell to proliferate it must enter the S-phase of the cell cycle. Entry into S-phase requires the activities of CDK2 and CDK4, and these are negatively controlled by p21 and p27 (65). In addition to its negative effects on CDK activity p21 can also block DNA replication by binding PCNA (66). The anti-proliferative effect of IFN-γ may reflect its ability to induce the CDK inhibitors p21 and p27 (67), as well as repress CDK2 expression (68). Induction of p21 by IFN-γ occurs in a STAT1- and IRF-1-dependent manner (69-71). The p21 promoter contains three potential GAS sites allowing for a rapid increase in p21 mRNA following treatment with IFN-γ. Regulation of p21, p27, and CDK2 by IFN-γ provides multiple pathways through which IFN-γ mediates its anti-proliferative effects although this has not been proven *in vivo*.

IFN-γ can also inhibit cell proliferation through its effects on protein synthesis through non-specific mechanisms such as PKR and RNase L. Active PKR and RNaseL combine to degrade RNA nonspecifically and thus decrease proliferation due to inhibition of protein synthesis. Thus, various ways exist for IFN-γ to inhibit cell proliferation and these are not necessarily mutually exclusive.

d) Leukocyte-endothelium interactions. In inflammation, leukocytes are attracted to and extravasate through activated endothelium in postcapillary venules. The mechanism of leukocyte recruitment and extravasation is a complex, multi-step process of which many steps are affected by IFN-γ. Leukocyte transmigration across endothelium can be divided into four major steps: rolling, activation, firm adhesion, and transmigration (72). During inflammatory conditions, endothelium in the affected area is activated and expresses surface adhesion molecules and chemokines in response to proinflammatory

cytokines such as TNF, IL-1, and IFN-γ. Adhesion molecules mediate the rolling and firm adhesion while chemokines are responsible for the activation step in the extravasation of leukocytes into the affected tissue.

IFN-γ can affect the extravasation of leukocytes through its effects on chemokine expression. Usually in synergy with TNF and IL-1β *in vivo*, IFN-γ induces expression of the following chemokines: CXCL9, CXCL10, CXCL11, and CCL5 (73). CXCL9-11 specifically recruit activated/memory T lymphocytes whereas CCL5 recruits activated T cells as well as other leukocytes to the site (74). Granulocytes are not as readily observed in sites where IFN-γ is abundantly expressed, and this may be because IFN-γ inhibits expression of granulocyte recruiting chemokines such as CXCL1, CXCL2, and CCL11 (73;75;76). Thus, through induction and inhibition of specific chemokines, IFN-γ modulates the recruitment of cells required for cellular immunity while inhibiting recruitment of cells that mediate allergic reactions.

Adhesion molecules are important in the extravasation of leukocytes across vascular endothelium. Despite the presence of ISRE and GAS sites in the promoter regions of ICAM-1 and VCAM-1, there is little evidence for IFN-γ being necessary for leukocyte extravasation *in vivo*. IFN-γR1 -/- kidney allografts demonstrate the presence of leukocytic infiltrate in the interstitium of the graft with cell numbers no different than in wild-type allografts (77). IFN-γ -/- mice are able to destroy tumour cells in the lung, and both IFN-γ -/- and IFN-γR -/- mice successfully kill mastocytoma cells injected into the peritoneal cavity (78) (and our own results presented here). Altogether these studies show that leukocytes are able to transmigrate across endothelium and epithelium in the absence of IFN-γ. Although the type of cells recruited to the affected site differs

depending on the presence or absence of IFN-γ, it is unknown how much the altered chemokine expression or differential expression of adhesion molecules contributes to this effect.

e) *Regulation of innate immunity.* IFN-γ plays a central role in the regulation of the innate immune response by increasing the ability to destroy intracellular pathogens. IFN-γ increases microbicidal activity of macrophages through effects on reactive oxygen intermediates such as hydrogen peroxide and NO, allowing them to eliminate intracellular or phagocytosed pathogens more efficiently (79). Similar effects to those on macrophages have also been observed in neutrophils (80). IFN-γ enhances the effector functions of antibodies and complement proteins by increasing expression of Fc receptors on monocytes/macrophages and NK cells, and the expression of complement proteins and complement receptors on macrophages (81;82). Thus IFN-γ activates innate immune cells for the efficient elimination of pathogens.

f) *Regulation of adaptive immunity.* IFN-γ also regulates multiple aspects of adaptive immunity. Class switch to IgG3 in humans, or IgG2a in mice is facilitated by IFN-γ through a direct effect on the B cell that requires B cell activation and is enhanced by IL-1 (83). The contraction of CD4 T cell effectors generated during the immune response is mediated through activation induced cell death (AICD), which requires IFN-γ (84). During viral infection, IFN-γ alters the immunodominance hierarchies of antigen-specific CD8 T cells (85). In this study IFN-γ-deficiency decreased the death rate of

antigen-specific CD8 T cells through an as of yet uncharacterized mechanism. Thus, IFN-γ effectively regulates the function of B cells, and CD4 and CD8 T cells.

1.6 INTERACTIONS BETWEEN IFN-γ AND OTHER CYTOKINES

The effects of IFN-γ can be enhanced or diminished by other cytokines. Proinflammatory cytokines including TNF, the IL-12 family, and IL-18 tend to enhance IFN-γ effects by way of synergy or by increasing IFN-γ levels. Negative regulation of IFN-γ can be mediated by IL-4 and IL-13, which form the basis of the Th1-Th2 paradigm, and also by immunosuppressive cytokines including IL-10 and transforming growth factor (TGF)-β.

a) Negative regulation of IFN-γ by immunosuppressive cytokines. The TGF-β family is a set of 3 pleiotropic cytokines and TGF-β1 mediates negative effects on T cell function. TGF-β1 is produced by all leukocytes and controls differentiation, proliferation and activation of leukocytes (86). The induction of IFN-γ by IL-12 is inhibited by TGF-β1 (87). This is exemplified in the inflammatory disease of TGF-β1 -/- mice which displays excessive IFN-γ effects (88). IFN-γ and IL-12 also serve as antagonists of TGF-β1 *in vivo* and *in vitro* (89), although IFN-γ -/- mice do not display excessive TGF-β1 expression. The interplay between IFN-γ and TGF-β results in further changes in

immune responses due to the effects of TGF-β1 on DC viability, as a limiting factor in B cell expansion, and numerous inhibitory effects on macrophage function (86).

IL-10 negatively regulates IFN-γ effects through its potent deactivating effects on APCs. One of the principal effects of IL-10 is the dampening of proinflammatory cytokine production by APCs, which in turn decreases the ability of these cytokines to enhance IFN-γ production (90). IL-10 also deactivates the respiratory burst induced in macrophages by IFN-γ (91).

b) Positive regulation of IFN-γ by proinflammatory cytokines. In *in vivo* inflammatory states IFN-γ is likely never produced alone but in concert with many other proinflammatory cytokines including TNF. Certain actions of IFN-γ can be induced by TNF to some degree, and these two cytokines often synergize to produce a stronger effect. The synergistic effect involves IFN-γ-induced transcription factors in combination with NF-κB; the principal mediator of TNF-induced transcription. Many genes are inducible by IFN-γ and TNF due to the presence of ISRE or ISRE-like sites and NF-κB sites in their promoters. Further, TNF can also enhance IRF-1 expression by acting through the NF-κB sites present in the IRF-1 promoter (92). IFN-γ in turn enhances TNF responses by inducing IκB phosphorylation leading to activation of NF-κB and enhancing expression of the TNF receptor (93;94). The synergy between IFN-γ and TNF is dominated differently depending on the context of the response. For example, in cytotoxicity, IFN-γ seems to potentiate TNF, whereas in MHC regulation the primary role is for IFN-γ but potentiated by TNF.

Members of the IL-12 family are proinflammatory cytokines that promote IFN-γ effects by increasing IFN-γ production in T cells and NK cells. IL-12, -23, and -27 work in concert to promote the differentiation of Th1 cells and thus increase IFN-γ production (see below).

1.7 TH1 AND TH2 T HELPER CELL POLARIZATION

Naive CD4 T cells can differentiate into Th1 or Th2 effector cells which are distinguished by the array of cytokines that they express. Th1 cells are characterized by the production of proinflammatory cytokines promoting macrophage activation and the control of intracellular pathogens. Th2 cells produce cytokines that promote allergic responses and the elimination of parasites and extracellular pathogens. The differentiation into Th1 or Th2 effector cells is regulated by APC-derived cytokines, unique transcription factors induced in the differentiating T helper cells, and perhaps by unique DC subtypes.

a) The Th1/Th2 paradigm. Mosmann and Coffman characterized the polarization of mouse CD4 T helper clones into two distinct subsets based on their cytokine profiles (95). Mouse Th1 cells produce IFN-γ, IL-2, and lymphotoxin whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (96). Highly polarized Th1 and Th2 cytokine patterns are generally only found following many weeks of *in vitro* stimulation or in cases of persistent infections *in vivo* (95;97). Thus, strictly defined Th1 and Th2 cytokine

patterns are not observed during primary immune responses. The initial activation of naive CD4 T cells by APCs leads to production of IL-2, in the absence of IL-4 or IFN-γ, causing the proliferation of the responding cells prior to their differentiation into Th1 or Th2 cells. Th1 and Th2 differentiation patterns are most likely the extremes of differentiation in the full spectrum of possibilities during an immune response. Thus, while the Th1-Th2 paradigm provides a framework for the study of various models, in most cases it does not reflect the full complexity of cytokine regulation by T cells *in vivo*.

The initiation of Th1 and Th2 responses requires the presence of specific cytokines required to drive this differentiation. Th1 differentiation is directed by IL-12 family of cytokines and IFN-γ. DC and macrophages produce IL-12 family members upon activation by microbial products such as LPS. In turn, recently activated, uncommitted CD4 T cells express IL-12 receptors whose expression is maintained along the Th1 differentiation pathway, but lost in Th2 cells (98). IFN-γ produced early in the immune response is from NK cells. NK cells are actively recruited to draining lymph nodes in a CCR7-independent, CXCR3-dependent manner where they produce IFN-γ, likely in response to proinflammatory cytokines such as IL-12 (2). *In vitro* differentiation of Th2 cell requires exposure of naive cells to IL-4 at the initiation of the immune response along with IL-2 (99). The cell type responsible for the IL-4 early in the response remains unknown.

b) Regulation of Th1-differentiation and IFN-γ production by proinflammatory cytokines. IFN-γ production by T cells depends on TCR-mediated T cell activation. However, T cells activated during an immune response are in the presence of a

combination of proinflammatory cytokines that synergistically enhance IFN-γ production by T cells. The most potent inducers of IFN-γ include the IL-12 family of cytokines (IL-12, IL-23, and IL-27) and IL-18. IL-18 and the IL-12 family of cytokines are produced mainly by mature DC and activated macrophages and promote IFN-γ production from T cells and NK cells, as well as Th1 differentiation (100).

The IL-12 family of cytokines. The IL-12 family of cytokines work in concert to induce Th1 responses. The effects of IL-12 on newly activated T cells are dramatic, yet IL-12 cannot act on naïve T cells due to their lack of IL-12 receptor expression. Expression of the IL-12Rβ2 chain can be upregulated by IL-2 on CD4 T cells (101), and especially by IL-27 (102). This has led to a proposed sequential mechanism of action in the regulation of CD4 T cell differentiation: Since the IL-27 receptor is selectively expressed on naïve T cells, and IL-27 is the first IL-12 family cytokine produced by DC, IL-27 likely primes naïve T cells to begin Th1 differentiation by increasing IL-12Rβ2 and T-bet expression. Once primed by IL-27 to receive IL-12, activated CD4 T cells continue Th1 differentiation. IL-23 acts on effector/memory CD4 T cells to sustain long-term Th1 responses.

IL-12 is the prototypical IFN-γ-inducing cytokine produced mainly by mature DC and activated macrophages. It is the ability of IL-12 to induce the differentiation of newly activated and resting memory CD4 T cells to Th1 cells, and provide co-stimulatory signals for maximal IFN-γ production (103) which promotes Th1 responses. Although IL-12 is essential for optimal Th1 responses, Th1 responses can be generated in its absence (104), likely due to the presence of the other IL-12 family members.

The functions of IL-23 are similar but not completely overlapping with those of IL-12. IL-12 and IL-23 can both induce IFN-γ production and drive Th1 differentiation. The principal difference between IL-23 and IL-12 is the ability of IL-23 to act selectively on effector/memory T cells. Memory and effector T cells generated during the immune response are non-respondent to IL-27 or IL-12. However, IL-23 induces strong proliferation of memory CD4 T cells yet has no effect on naïve CD4 T cell proliferation (105). *In vitro*, IL-23 can promote the production of IL-12 from DC and enhance productive DC antigen presentation (106), which may allow further IL-23 mediated regulation of T cell function through modulation of DC.

In contrast to IL-23, IL-27 induces proliferation of naïve but not memory CD4 T cells (107). The importance of IL-27 in Th1 differentiation is evident in IL-27R -/- mice which develop impaired Th1 responses and are more susceptible to *Listeria* infections (108). An important effect of IL-27 in initiating Th1 responses is the induction of the Th1-specific transcription factor T-bet as well as the suppression of the Th2-specific transcription factor GATA-3 (102).

IL-18. IL-18 is a member of the IL-1 cytokine family that strongly induces IFN-γ production. The induction of IFN-γ production from T cells by IL-18 is more potent than that by IL-12 and was therefore originally named IFN-γ-inducing factor (8). IL-18 is secreted by a variety of cells including macrophages, DC, and epithelial cells (109). The IL-18 receptor complex can be up-regulated on naive T cells, Th1 cells, and B cells by IL-12 (110). Thus IL-18 works in conjunction with IL-12 to further increase IFN-γ production. Cooperation between IL-12 and IL-18 is evident in their signaling pathways:

IL-12-induced STAT4 has been shown to enhance IL-18-induced AP-1 binding to and activation of the IFN-γ promoter (111).

c) The role of transcription factors in Th1-Th2 differentiation. Transcription of the IL-4 and IFN-γ genes is dependent on ubiquitous transcription factors such as NF-AT and AP-1 but the restricted expression of IL-4 and IFN-γ is mediated by the cell-type specific transcription factors GATA-3 and T-bet, respectively.

IFN-γ transcription and Th1 differentiation in CD4 T cells are regulated by the transcription factor T-bet. T-bet is a potent transactivator of the IFN-γ gene and its ectopic expression can lead to IFN-γ production in non-IFN-γ producing cells (112). Expression of T-bet correlates with IFN-γ expression in CD4 T cells, NK cells, and B cells and is absent in polarized T helper cells that do not express IFN-γ. IFN-γ treatment further induces T-bet expression creating an autocrine positive feedback loop for IFN-γ production (17). Interestingly, unlike CD4 T cells and NK cells, T-bet expression is not required for IFN-γ production by CD8 T cells (112). Instead, CD8 T cells require the transcription factor eomesodermin for proper IFN-γ production (113).

Transcription of Th2 cytokines is under the direction of two transcription factors: GATA-3 and c-Maf. GATA-3 is a transcription factor that can directly regulate IL-4, IL-5, and IL-13 and thus serves as a global Th2 cytokine regulator (114). The proto-oncogene c-Maf is specific and critical for high levels of IL-4 production but is unable to initiate transcription on its own (115).

The Th1- and Th2-specific transcription factors also play antagonistic roles that may enhance those displayed by their respective cytokines. GATA-3 is capable of

inhibiting production of IFN-γ and Th1 development, possibly by repressing expression of IL-12Rβ2, in an IL-4-independent manner (116). Ectopic T-bet expression in Th2 polarized cells represses IL-4 and IL-5 and converts these into IFN-γ-producing cells (15). The antagonistic roles of these transcription factors may underlie the antagonism observed with the cytokines or at least enhance those effects.

1.8 IFN-γ-, IFN-γ RECEPTOR-, AND IRF-1-DEFICIENT MICE

a) IFN-γ and IFN-γ receptor-deficient mice. Many advances in understanding the function of IFN-γ *in vivo* were a direct result of the generation of IFN-γ -/- and IFN-γR -/- mice. IFN-γ -/- and IFN-γR -/- mice are viable, and develop normally. Most importantly, these mice are immunocompetent, making them very useful for immunological studies. IFN-γ -/- and IFN-γR -/- mice have been useful tools in elucidating the effects of IFN-γ on immune and non-immune cells. Lymphoid organs of IFN-γ -/- mice are histologically similar to wild-type mice, showing no differences in the number of splenocytes or thymocytes. CD4 and CD8 T cells, and B cell numbers in spleen and thymus are also not affected by the absence of IFN-γ (117). Thus, IFN-γ and IFN-γR are not necessary for the development of the immune system. These results suggest that the immunoregulatory aspects of IFN-γ had been overstated at least with respect to T cell function.

Although development of the immune system is normal in IFN-γ -/- mice, differences observed highlight critical requirements for IFN-γ. IFN-γ -/- splenocytes display excessive proliferation in response to concanavilin A as well as in mixed

leukocyte cultures (MLCs) demonstrating the anti-proliferative effects of IFN-γ. Of special interest to our project, IFN-γ -/- responders in MLC generate enhanced lytic activity against allogeneic targets compared to wild-type responders (118;119). This helped to demonstrate that IFN-γ is not required for CTL generation during the MLC and may actually suppress the response to allogeneic cells. NK cells from IFN-γ -/- mice, unlike CTL, demonstrated reduced resting lytic activity showing that IFN-γ is required for basal NK activity. IFN-γ is required for NOS-2 and NO production by macrophages as well as superoxide anion production in response to mycobacterial infection (118). In our lab the phenotype of IFN-γ -/- mice has been characterized to include profound defects in MHC regulation, altered alloimmune responses, and altered patterns of graft rejection (18;59;77;120;121).

The phenotype of IFN-γR1 -/- mice is similar to those of IFN-γ -/- mice with the obvious difference that they are unresponsive to IFN-γ. IFN-γR1 -/- mice have defects in infectious immunity to Mycobacteria and Listeria, to certain viruses including Vaccinia (26;117). As predicted, IFN-γR1 -/- mice display reduced IgG2a production (122). The similarities between IFN-γR1 -/- and IFN-γ -/- mice demonstrate the non-promiscuous interaction between IFN-γ and its receptor.

b) IRF-1-deficient mice. The phenotype of IRF-1 -/- mice is more drastic than that of IFN-γ -/- or IFN-γR -/- mice. IRF-1 -/- mice display a dramatic decrease in their CD8 T cell population as well as a thymocyte developmental defect revealing a critical role for IRF-1 in T cell development (123). Defects in MHC regulation are also present in IRF-1 -/- mice as well as an altered pattern of rejection when IRF-1 -/- mouse kidneys are

transplanted into allogeneic wild-type hosts (54;124). The CD8 T cell deficiency in these mice is thought to be linked to the requirement of IRF-1 for proper MHC class I expression (125).

1.9 GENERATION OF T CELL RESPONSES

a) Overview of effector T cell generation. T cell activation is a crucial step in the generation of immune responses to specific antigens. T cell responses are initiated in SLOs including lymph nodes, spleen, or Peyer's patches where naïve T cells; which have not yet encountered their cognate antigen, reside. The trafficking pattern of naïve T cells is restricted to SLOs, and they are unable to enter peripheral tissues. Naïve T cells traverse from blood into SLOs, where they pass through the T cell areas in search of antigen, enter lymph vessels, return to the blood and enter other SLOs.

Within SLOs naive T cells interact with DC that have migrated from the periphery in response to infection or injury. Resident DC in peripheral tissues remain in an immature state in the absence of infection or injury. Immature DCs endocytose material from their surroundings for surveillance and sense environmental disturbances in the peripheral tissues. They respond to disturbances characterized by pathogen associated molecular patterns which are recognized through toll-like receptors. Upon toll-like receptor activation, DC mature into potent APCs, and migrate to SLOs. The migration of immature DCs to SLOs on the other hand helps to maintain tolerance (126).

Thus DCs interpret their environment and determine whether the endocytosed antigen should lead to an immune response, no response, or tolerance.

The movement of DCs and the movement of naive T cells are co-ordinated to bring them into contact with each other to ensure T cell activation. Upon encountering their cognate antigen presented on mature DC, naive T cells become activated. Following activation, CD4 T cells move to the periphery or to B cell regions/lymphoid follicles of SLOs to provide cytokines and costimulation to B cells (127), permitting them to class switch and to become antibody producing plasma cells. Activated CD4 and CD8 T cells and B cells leave SLOs after many rounds of cell division as effector cells capable of entering peripheral tissues to apply their effector functions.

CD4 T cells can become effector cells but also are important regulatory cells in the generation of nearly all forms of immunity and the prevention of autoimmunity. They exert their effects through the production of cytokines and by direct contact with other cells. Regulatory T cells (CD4+25+, FoxP3+) are the primary type of CD4 T cell whose function is regulatory instead of effector (128).

As the host defense challenge subsides, the majority of T cells generated in an immune response will be deleted. T cells are deleted through cell death, either AICD triggered by antigen, or cell death resulting from antigen withdrawal. Programmed cell death spares only sufficient numbers of antigen-specific memory cells to maintain the capacity to produce an efficient rapid response upon a secondary exposure to the same antigen.

1.10 INTERLEUKIN-2

IL-2 is an important growth factor for T cells that is quickly expressed following T cell activation. Upon allogeneic stimulation, IL-2 is primarily produced by CD4 T cells (129) and increases the proliferation of CD4 and CD8 T cells.

a) Induction of IL-2 gene expression. Naïve CD4 T cells produce IL-2 quickly following activation and proliferate. During the proliferation, CD4 T cells also differentiate and become capable of producing effector cytokines such as IFN-γ and IL-4 (130). IL-2 is unique amongst CD4 T cell cytokines in its ability to be expressed without the need for cell division. Expression of *Il4* and *Ifng* requires cell cycle progression and differentiation in order to alter chromatin structure and allow for gene transcription (130). Chromatin remodeling of the *Il2* gene on the other hand occurs during commitment to the T cell lineage in the thymus allowing for immediate expression following activation (131). Although *Il2* can be expressed early upon activation of naïve CD4 T cells, previously activated CD4 T cells induce the *Il2* gene more strongly (131). Activated CD8 T cells produce IL-2 in much lower amounts compared to CD4 T cells, thus, effective CTL responses often require help from CD4 T cells for sufficient IL-2 other activating signals.

Transcriptional regulation of the *Il2* gene involves the coordinated actions of several transcription factors. The *Il2* promoter is composed of a 300 bp region upstream of the transcription start site (132). Many of the transcription factors required for *Il2* expression are also used for *Ifng* expression including NF-AT, AP-1, and NF-κB (133).

The regulatory elements in the *Il2* promoter include two binding sites for NF-AT and AP-1, and an NF-κB site (133). The octamer proteins also regulate *Il2* expression by binding to two octamer-binding sites within the promoter (134). Optimal production of IL-2 requires TCR stimulation, which activates the transcription factors mentioned above, as well as costimulation through CD28, which increases IL-2 mRNA accumulation. This effect has been mapped to the CD28 response element located in the *Il2* promoter which may involve AP-1 and NF-κB family members (132;135). Similar to regulation of IFN-γ production, *Il2* transcription depends on continued T cell stimulation (136).

b) The IL-2 receptor. Signaling in response to IL-2 is mediated through the IL-2 receptor which can be expressed as a low affinity or high affinity form. The high affinity IL-2 receptor is a trimeric complex composed of IL-2Rα, IL-2Rβ, and the common γ chain (γ_c) expressed on activated T cells. The low affinity IL-2 receptor is composed of the IL-2Rβ and γ_c and is expressed on resting T cells. Heterodimerization of the cytoplasmic domains of the IL-2Rβ chain and the γ_c are sufficient and necessary for signal transduction, but coexpression of the α subunit (CD25) is required to generate the high affinity IL-2 receptor. Naïve T cells lack CD25, but its expression is induced upon antigenic TCR stimulus (137). Binding of IL-2 initiates a signaling cascade through JAK1, JAK3, and STAT5, and promotes T cell proliferation (138;139). The importance of CD25 and IL-2 in the regulation of alloimmune responses is highlighted by the effects of a non-depleting monoclonal antibody against CD25. This antibody reduces the frequency of acute rejection and improves short-term graft survival in renal transplant patients (140).

c) *Positive and negative effects of IL-2 on T cell function.* IL-2 drives the antigen-dependent proliferation and survival of T cells and activates NK cells. Promotion of T cell proliferation and survival by IL-2 is due to the induced expression of anti-apoptotic proteins of the Bcl-2 family (141). It has long been known that IL-2 can mediate CTL differentiation *in vitro* (142) and is also commonly used to maintain CTL and NK cell lines. *In vivo* CTL responses to LCMV are reduced approximately three-fold in IL-2 -/- mice compared to WT (143), demonstrating an amplifying role for IL-2, despite not being absolutely necessary for CTL responses. Given its potent effects on CTL generation, IL-2 showed promise in tumor immunotherapy. Initial studies in mice were very promising (144), but recombinant IL-2 given to patients proved toxic due to excessive capillary leakage (145). Nonetheless IL-2 is approved for use to enhance T cell responses in patients infected with HIV and suffering from metastatic cancer (146;147).

Although IL-2 was originally characterized as a positive T cell growth factor, it can also negatively regulate T cell function. Activated CD4 T cells are rendered susceptible to Fas-mediated AICD by IL-2 (148). This is a result of increased cell surface levels of FasL and decreased expression of FLICE-like inhibitor protein; an inhibitor of Fas signaling (149). Further evidence for the negative effects of IL-2 stems from observations in IL-2 -/- and IL-2Rβ -/- mice which accumulate activated T cells and develop autoimmunity (150;151), indicating a central role for IL-2 in maintaining T cell tolerance.

1.11 CYTOTOXIC T LYMPHOCYTES (CTL)

CTL are cytotoxic effector cells of adaptive immunity critical for the eradication of intracellular pathogens. At times their potent cytolytic activity is activated when it is not desirable as is the case in allograft rejection and autoimmunity. Before a proper CTL response is mounted naïve CD8 T cell numbers must be greatly increased. To increase their numbers and develop effector function, CD8 T cells undergo several rounds of clonal expansion. Effector functions of CTL allow for the efficient destruction of infected/antigenic cells at the affected site. Some of these CTL effector molecules are shared with NK cells although only CTL are antigen-specific.

a) Generation of CTL responses. Naïve CD8 T cell expansion occurs in SLOs following encounter with antigen-bearing DC. DC that have been activated in the periphery acquire antigens from their surroundings and increase surface expression of costimulatory molecules and MHC class II and MHC class I molecules on which antigenic peptides are presented. In SLOs DC can interact with CD4 and CD8 T cells at the same time or sequentially but the presence of CD4 T cells during CD8 T cell priming is crucial for the generation of effective secondary responses (152). CD8 T cells undergo massive expansion required to generate sufficient numbers of CTL. CTL use cytolytic molecules and cytokines to destroy cells harboring intracellular pathogens. Following the initial expansion, CTL must undergo a contraction phase in which the majority of effector T cells are eliminated leaving behind a stable number of memory cells.

b) Differences between effector CD4 T cells and CTL. Although both CD4 and CD8 T cells expand in response to antigen, their requirements and kinetics of expansion differ. Naïve CD8 T cells require a shorter period of antigen stimulation to proliferate compared to naïve CD4 T cells (153). CD8 T cells also proliferate sooner and at a faster rate than CD4 T cells (154;155). Development of effector function (ie. the ability to produce IFN-γ or IL-4 for CD4 T cells and IFN-γ, TNF, and cytolytic activity for CD8 T cells) is also more readily attained by CD8 T cells (156). As a result of these differences, CD8 T cell responses are greater in magnitude than CD4 responses at least in response to intracellular pathogens (157;158). The generation of larger numbers of CTL effectors may underlie their requirement for direct cell to cell contact for their cytotoxic ability. CD4 T cells on the other hand, do not require cell to cell contact since their effector function is primarily mediated by the production of cytokines.

Following clonal expansion, CD4 and CD8 T cells undergo a contraction phase where the majority of the cells are deleted. Once again the mechanisms for the deletion of CD4 T cell effectors and CTL differ. CD4 T cell effector numbers are primarily reduced by Fas-mediated apoptosis (159), a process commonly referred to as AICD. CTL on the other hand are inherently resistant to Fas-mediated apoptosis (160), and thus their primary deletional mechanism is mediated through the TNF receptor (161). TNF related apoptosis-inducing ligand (TRAIL) was recently shown to mediate AICD in CD8 T cells primed in the absence of CD4 T cells (162), although what role this may play during normal CD8 T cell responses is unknown.

c) CD4 T cell requirement for CTL responses. CD4 T cells are required for the generation of proper CD8 T cell responses leading to generation of memory CTL. CD8 T cells are activated through interactions with DC that have been primed by previously encountering CD4 T cells. The details of this interaction are not completely understood but it is known to be mediated through CD40-CD40L (163). DC may also be primed for CD8 T cell activation by CD40 or by infection with certain viruses (164). Viral activation of DC allowing them to prime CTL autonomously can explain why CD4 T cells are not always required in certain immune responses to virus. Indeed, CTL effector function measured at the peak of certain immune responses are not affected by the absence of CD4 T cells (165;166). However, secondary responses to antigen are dependent on CD4 T cells in both T-help-dependent as well as T help-independent *in vivo* responses (152). CD8 T cells primed following depletion of CD4 T cells fail to respond upon secondary stimulation, but priming prior to CD4 T cell depletion does not affect secondary responses. This demonstrates that the capacity for secondary expansion is programmed and becomes cell-autonomous once CTL are primed in the presence of CD4 T cells.

1.12 TRANSPLANTATION

Transplants between nongenetically identical members of the same species leads to the activation of alloreactive T cells that destroy the transplant. The massive response is invoked due to the large number of naive and memory T cells capable of recognizing

allogeneic MHC. Thus antigen presentation and MHC differences drive alloimmune responses. Unlike most responses to cognate antigens where approximately $1/10^6$ of the total T cells may respond, up to 5% of the T cell population may respond to an allogeneic stimulus.

a) Antigen presentation. Allografts induce alloimmune responses due to the recognition of nonself antigens from the graft by recipient T cells. Recipient T cells may encounter alloantigen through direct or indirect pathways.

Indirect antigen presentation is the way antigen is normally presented in immune responses. In the indirect pathway antigen is taken up by APCs, processed, and presented as peptides on MHC molecules. In alloimmune responses, graft-derived cells or released antigens can be taken up by recipient APCs. Recipient APCs process donor MHC and present donor-derived peptides on recipient MHC molecules to T cells initiating the alloimmune response (167).

Direct antigen presentation involves the recognition of intact donor MHC on the surface of donor cells. Donor APCs may migrate from the graft to recipient SLOs and activate alloreactive T cells to initiate the alloimmune response. Direct antigen presentation also occurs when recipient T cells encounter the allograft and recognize donor MHC. B cells also recognize intact donor MHC antigen by their B cell receptors leading to alloantibody production (168). Direct and indirect antigen presentation both likely occur in immune responses to allografts.

b) MHC class I and class II differences. The relative importance of MHC class I versus MHC class II differences in transplantation depends on the organ transplanted and the species. In humans, MHC class I and II incompatibilities can contribute to graft outcomes (169). In murine models of transplantation, rejection is associated with donor MHC class I and host MHC class II in special cases. For example, in a heart transplant rejection model across an MHC class II difference, CD4 T cells required donor class II but not host class II, suggesting that the CD4 effector T cells must directly engage donor MHC class II, independent of the role of host class II (170). However, rejection of mouse kidney allografts is relatively normal when grafts lack MHC class II, although the mononuclear infiltrate is faintly reduced (171). The current consensus is that in rejection in humans and in mice, host CD4 T cells recognizing donor class II or host class II play an important role as helpers for effector T cell generation and for alloantibody production by B cells. However, most typical T cell mediated rejection is dominated by CD8 T cells (172), suggesting that CD8 T cells directed against donor class I incompatibilities are important in allograft rejection.

c) Initiation of alloimmune responses. The donation and transplantation process induces many types of injury in the graft and host tissues. These injuries can themselves induce inflammation including MHC induction, cytokine production, altered gene expression in the stressed tissues, and increased chemokine expression (173;174). Injury increases MHC expression in the transplant at least in part by triggering IFN-γ production, but much of this is independent of IFN-γ. It is not clear how important this injury response is in triggering alloimmunity. However, the pattern of activation of

innate immunity in response to tissue stress followed by adaptive immunity is typical in the immune response (175). The trigger for innate immunity in transplantation is not clear, but one consequence could be APC activation and maturation in response to tissue damage resulting from ischemia/reperfusion and surgery.

d) Generation of T cell effector functions in alloimmune responses. The generation of effector T cells is thought to be initiated in the same manner as in immune responses to pathogens. Naive T cells may be triggered by donor or recipient APCs to proliferate and develop effector functions in SLOs. Memory cells may also be activated in SLOs in the same manner or they may become activated by recognizing cells in the allograft directly. During allograft rejection in human adults both naïve and memory populations are probably activated simultaneously.

T cell proliferation generally occurs in SLOs and is mostly driven by T cell growth factors such as IL-2 and possibly IL-15. Proliferation of alloreactive clones generates a considerable number of responding CD4 and CD8 T cells in SLOs which leave upon acquiring effector function. The exit from lymph nodes is due at least in part to the change in chemokine receptors expression induced by effector differentiation. Five days following transplantation, mononuclear cells are visible in the interstitium of the allograft {18157}.

e) Events in the rejecting allograft. Activated donor-specific T cells and B cells enter the graft and enhance inflammation leading to increased cellular infiltrate entering the graft. Many cells in the graft express and present MHC class I and class II to

alloreactive T cells. IFN-γ is strongly expressed during rejection and further induces MHC expression in vascular endothelial cells, epithelial cells, and parenchymal cells in the graft. Changes in the graft endothelium induced by injury may facilitate entry, and the subsequent changes are induced by the ongoing immune response in the tissues. Whether T cell proliferation occurs in the graft or the extent to which proliferation in the graft may be needed, as opposed to T cell proliferation in SLOs, is not clear. Effector systems are detectable in the graft along with the mononuclear infiltrate. Expression of IFN-γ, TNF, and other cytokines with effector function, as well as CTL associated molecules perforin, granzymes A and B are all observed.

The pathogenesis of immune mediated tissue injury in rejection is unknown. The typical early rejection episode is mediated primarily by effector T cells, and many of its features are unchanged in mice lacking immunoglobulin (176). Although CTL genes are strongly expressed, perforin-deficient mice still reject despite lacking the ability to lyse allogeneic targets in short term assays. This suggests that CTL molecules are not essential in mediating the injury to transplanted tissues (177). Thus CTL are associated with the rejection, but the effector functions used by the CTL in allograft rejection are not established. Nevertheless direct contact between T cells and target tissues is observed, and correlates with clinical functional deterioration in solid organ allografts. It seems likely that T cell mediated invasion and injury of the allograft are critical events in T cell mediated graft rejection, and reflect T cells engaging donor cells even if the mechanism of injury is not dependent on known cytotoxic mechanisms.

1.13 RATIONALE AND HYPOTHESIS

Allograft rejection is associated with the presence of CTL and abundant IFN-γ production. CTL in the allograft are fully differentiated effectors expressing cytotoxic molecules and effector cytokines such as IFN-γ. IFN-γ is also expressed in SLOs where CD4 and CD8 T cells differentiate into effector cells. Many of the immunoregulatory aspects of IFN-γ are involved with regulation of lymphocyte function and survival. Immunoregulatory mechanisms of IFN-γ on CTL generation are therefore likely involved in the mechanism of allograft rejection. However, allograft rejection in IFN-γ -/- hosts is accelerated rather than attenuated suggesting a negative role for IFN-γ. Based on our preliminary studies we hypothesized that IFN-γ negatively regulates CTL generation in alloimmune responses. We examined CTL generation in MLC following allogeneic tumor cell challenge. To address this hypothesis we proposed several questions:

1. How does IFN-γ limit CTL generation?
2. Is the effect of IFN-γ on CTL generation mediated through IRF-1?
3. Is CTL generation limited *in vivo* as well as *in vitro*?
4. Does IFN-γ promote the elimination of allogeneic tumor cells?

These questions are addressed in the following chapters. The answers contribute to the further understanding of immunoregulation by IFN-γ and may be applicable to other inflammatory diseases where regulation of CTL generation is important.

1.14 FIGURES

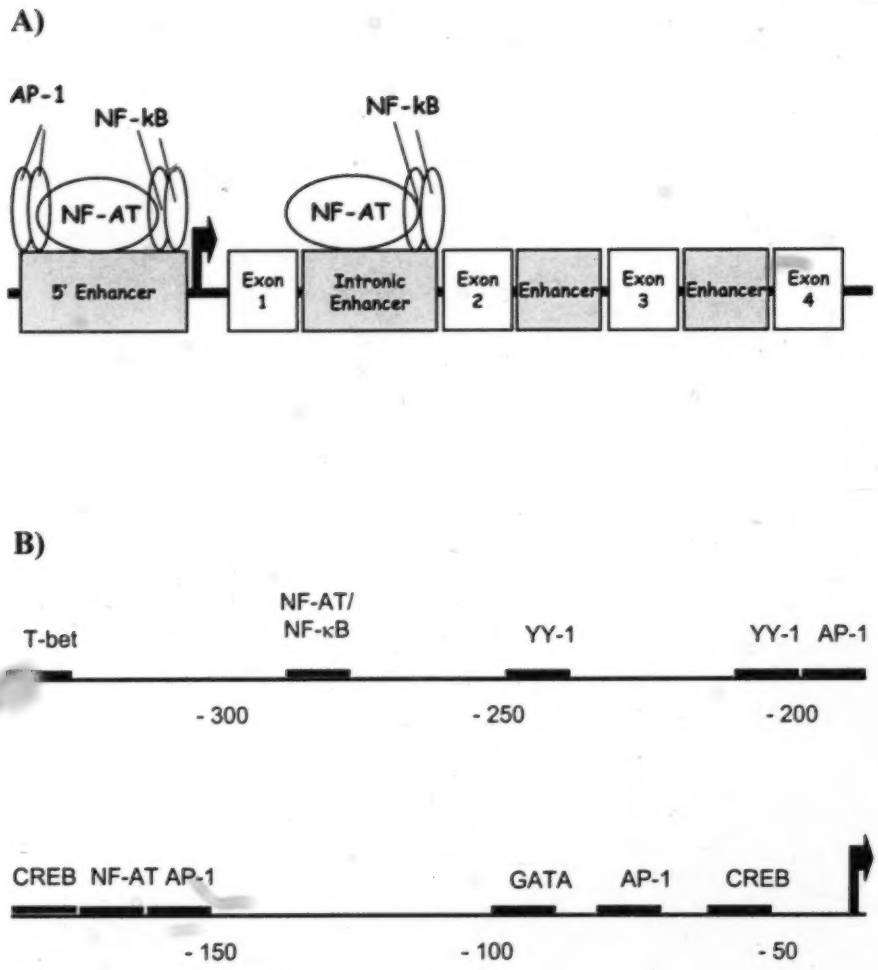

Figure 1 a: Architecture of the IFN-γ gene and promoter. A) The IFN-γ gene is composed of four exons. Introns in the 5' untranslated region and between the exons contain enhancers that allow binding of the transcription factors AP-1, NF-AT, and NF-kB. B) Outline of the 5' region of the IFN-γ promoter highlighting the sites for specific transcription factor binding.

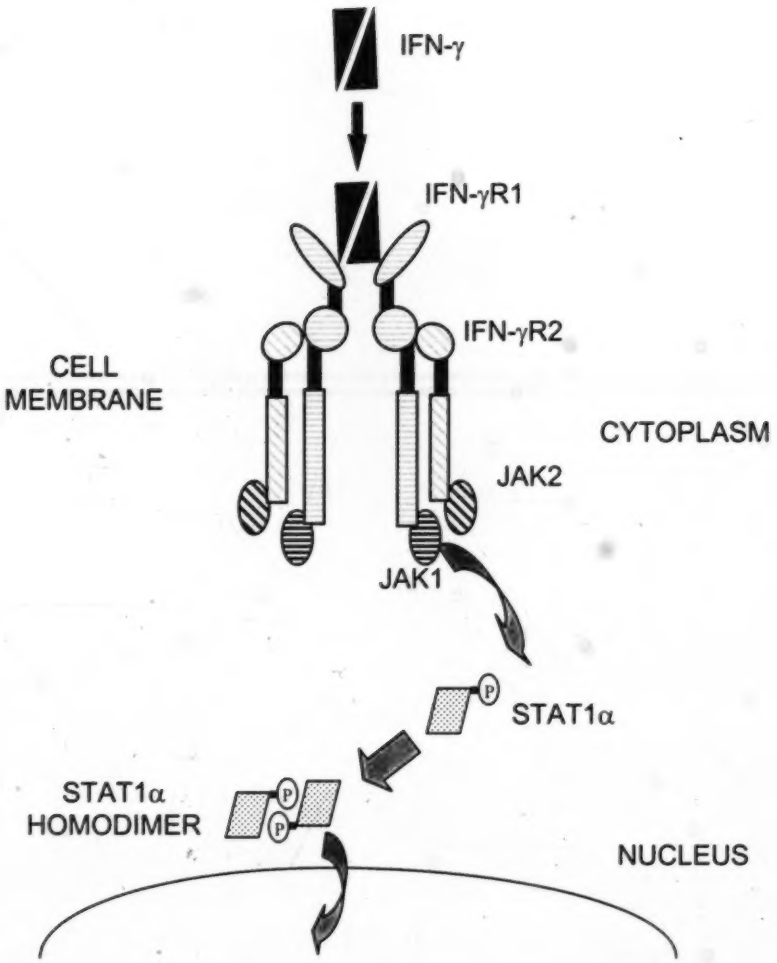

Figure 1 b: Signal transduction from the IFN-γ receptor. IFN-γ is initially bound by two IFN-γ-R1 chains followed by recruitment of two IFN-γR2 chains. JAK1 and JAK2 phosphorylate STAT1α monomers which then form homodimers and enter the nucleus.

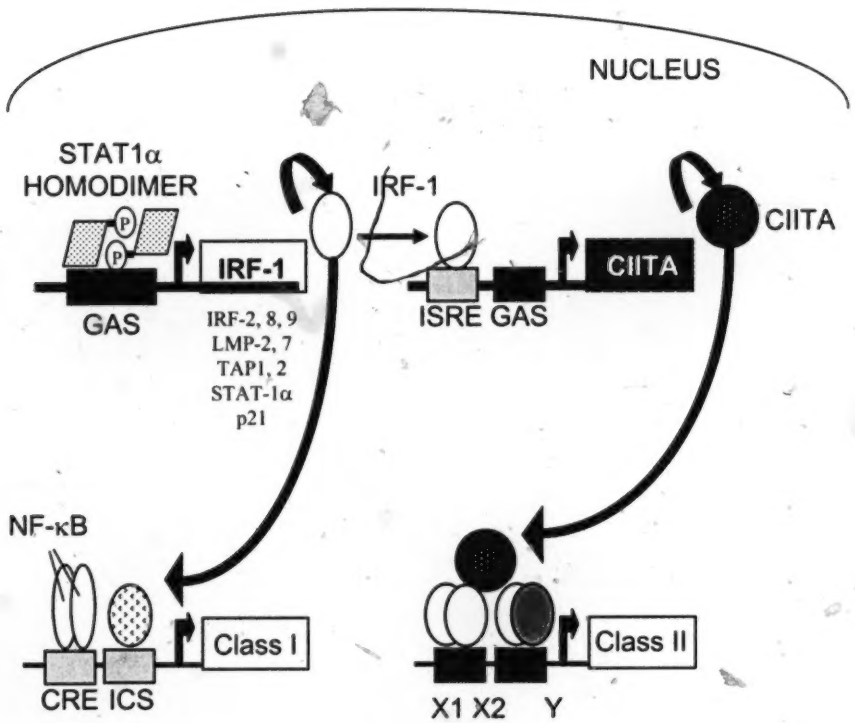

Figure 1 c: Gene transcription of secondary and tertiary transcription factors in response to IFN-γ. STAT1 homodimers bind to GAS sites on the promoters of secondary transcription factors (eg. IRF-1). IRF-1 binds ICS sites on the promoters of IFN-γ-inducible genes including tertiary transcription factors.

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CHAPTER 2

MATERIALS AND METHODS

2.1 INTRODUCTION

In the studies described herein, we utilized a number of different techniques and gene-deficient mice. Given that the focus of our studies is IFN-γ, we were fortunate to have access to IFN-γ -/-, IFN-γR1 -/-, and IRF-1 -/- mice. The generation and maintenance of these, and wild-type control mice, is described below. Mouse cell lines were used either as target cells in ^{51}Cr release assays or to induce *in vivo* alloimmune responses. Details of the cell lines and their maintenance are also described.

Cells isolated from mouse spleen and lymph nodes were used in *in vitro* experiments and to assess *in vivo* immune responses. *In vitro* studies mainly involved the generation of CTL in MLC while assessing cytokine production in the process. Cell isolation and MLC preparation procedures are described below. Cytokine production was assessed by ELISA or intracellular cytokine (IC) staining. External manipulations to the cultures included the addition of recombinant cytokines or antibody neutralization of endogenous cytokines. Functional assays from MLC cells included ^{51}Cr release assays to assess the generation of CTL activity and MTT assays to determine proliferation. Activation state of the effectors generated in MLC was determined by surface expression of activation markers by flow cytometry and by expression of granzyme B mRNA, assessed by real-time RT-PCR.

The strength of *in vivo* alloimmune responses was determined using some of the techniques used in our in vitro studies. Flow cytometry was again used to examine the activation state of CD8 T cells, determine the number of macrophages and granulocytes. Restimulation assays were used to determine the strength of the allospecific responses generated as a readout of cytokine production as determined by ELISA.

2.2 MATERIALS AND METHODS

IFN-γ -/- BALB/c mice. The original IFN-γ -/- mice were created by disrupting the IFN-γ gene, inserting a neomycin-resistance gene, and replacing one copy of the wild-type gene in embryonic stem cells by homologous recombination. These stem cells were used to construct mice heterozygous for the disrupted gene, which were intercrossed and the progeny were selected for homozygosity (1). Heterozygous BALB/c and BALB/c mice with intact wild-type IFN-γ genes were provided to us as a generous gift from Dr. Tim Stewart (Genentech, South San Francisco, CA). Heterozygous BALB/c mice were intercrossed (Health Sciences Laboratory Animal Services (HSLAS), University of Alberta) and homozygous IFN-γ -/- mice were identified by tail skin DNA testing. Homozygosity of the offspring was confirmed by further tailskin testing. All IFN-γ -/- mice used were confirmed homozygous (confirmation done by Joan Urmson).

IFN-γR1 -/- 129/B6 mice. The original IFN-γR1 -/- (129/Sv/Ev) mouse strain with disrupted IFN-γR1 genes was generated by gene targeting in murine embryonic stem

cells (2). The IFN-γR1 gene was disrupted by inserting the neomycin-resistance gene into exon V, which encodes an extracellular domain. Homozygous 129/Sv/Ev mice were kindly provided to us through Dr Michael Aguet (University of Zurich, Zurich, Switzerland). A breeding colony is housed in the HSLAS facility at the University of Alberta.

IFN-γ -/- and IFN-γR1 -/- C57BL/6 mice. Homozygous IFN-γR1 -/- and IFN-γ -/- mice on a C57BL/6 background aged 6-8 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME).

Wild-type C57BL/6 mice. BALB/c mice with wild-type IFN-γ and IFN-γR1 genes aged 6-8 weeks were obtained from HSLAS at the University of Alberta (BALB/cCr//AltBM). C57BL/6 mice and 129/SvJ mice with wild-type IFN-γ and IFN-γR1 genes aged 6-8 weeks were purchased from The Jackson Laboratory. The mice were maintained in the HSLAS animal colony. All experiments conformed to approved animal care protocols.

Cell lines. The P815 mastocytoma cell line was acquired from the American Type Culture Collection (ATCC, Rockville, MD). The murine T lymphoma cell line RDM4 was kindly provided by Dr. K. Kane (University of Alberta). T6SV cells; CBA/J-derived, SV40 transformed cells, were obtained from The Jackson Laboratory. P815 and T6SV cells were grown in DMEM high glucose containing 10% FBS and 1% antibiotic-antimycotic (Invitrogen, Burlington, ON, Canada). RDM4 cells were grown in RPMI

media containing 10% FBS, 1% antibiotic/antimycotic (Invitrogen), and 50 μM β-mercaptoethanol.

Mixed Leukocyte Culture (MLC). MLCs were performed using splenocytes and lymph node cells from male BALB/c or IFN-γ -/- BALB/c mice as stimulators, and splenocytes and lymph node cells from male C57BL/6J, or IFN-γ-/- C57BL/6, or IFN-γR1 -/- C57BL/6 mice as responders. Mice were between 6-12 weeks of age at harvest. Spleens and lymph nodes (inguinal, axillary, brachial, and submandibular) were collected and single-cell suspensions prepared by pressing against a steel mesh, followed by red blood cell lysis using lysis buffer (0.15 M NH_4Cl, 1 mM $NaHCO_3$, 0.1 mM EDTA). Stimulator cells were treated 25 μg/ml mitomycin C (Sigma, St. Louis, MO) and incubated for 20 minutes at 37 °C. Responders and stimulators were added to 25 cm^3 flasks, 24-well plates, or 96-well plates for proliferation assays, at a concentration of $5x10^6$ cells/ml of complete RPMI media (10% FBS, 1X antibiotic/antimycotic (Invitrogen), 1 mM nonessential amino acids, 1 mM sodium pyruvate (Flow Laboratories, McLean, VA), and 50 μM β-mercaptoethanol.

Intracellular Cytokine (IC) Staining. IC staining procedure was started during the last six hours of the assay underway. Six hours prior to harvest Monensin (1X, eBioscience, San Diego, CA) was added to cultures. At harvest, cells were stained with anti-CD3ε (clone 145-2C11), and anti-CD4 or anti-CD8 (clones RM4-5 or 53.7-8, respectively, eBioscience) in PBS containing 5% FBS and monensin, and washed before fixation in 100 μl IC Fixation Buffer (eBioscience) for 20 minutes in the dark, at room temperature.

Following fixation, cells were washed in 2 ml Permeabilization Buffer (eBioscience). Cells were resuspended in 100 µl Permeabilization Buffer containing fluorochrome conjugated antibody to the intracellular cytokine (JES6-5H4 for IL-2 or XMG1.2 for IFN-γ, eBioscience), or rat isotype control IgG.

MTT Proliferation Assay. MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma Chemicals) was added to cells from MLCs after 44 and 92 hours of culture at a concentration of 0.5 mg/ml and incubated for 4 hours at 37°C. Assays were performed in a 96-well plate in triplicate, using 5 X 10^5 stimulators and 5x10^5 responders per well. At the end of the incubation, media was removed using a syringe and 28G needle and 200 µl DMSO was added to each well. After a minimum of 60 minutes of incubation at 37°C, absorbance at 570 nm was measured on a SPECTRAmax PLUS^{384} plate reader (Molecular Devices, Sunnyvale, CA) using 630 nm as a reference wavelength.

^{51}Cr Release Assay. Cytolytic activity generated *in vivo* or in an MLC was determined in a standard 4-hour ^{51}Cr-release assay. Target cells (P815 (H-2^d), RDM-4 or T6SV (H-2^k)) were labeled with 100 µCi of Na^{51}Cr in a 200 µl volume of 5% FBS/RPMI for 60 minutes at 37°C. Effectors were collected at the indicated times and incubated with ^{51}Cr-labeled target cells as indicated, at Effector:Target ratios of 3:, 10:, and 30:1, in a final volume of 0.2 ml of 5% FBS/RPMI, in 96-well V-bottom plates. After four hours at 37°C, 50 µl of supernatant was transferred to a fresh 96-well plate, and 150 µl of scintillant (OptiPhase SuperMix; Wallac, Loughborough, England) was added to each

well. The plates were analyzed in a beta counter (MicroBeta; Wallac). Spontaneous ^{51}Cr release from target cells was determined by incubation of target cells in the absence of CTL. Maximum ^{51}Cr releasable was determined by incubation in the presence of 2% Triton X-100 (ICN Biomedicals, Irvine, CA). Percent lysis was calculated as *% lysis = 100 X (experimental release – spontaneous release)/(maximum release – spontaneous release).*

ELISA Assay. Cytokine levels were quantitated in cell culture supernatants by ELISA using detection kits for mouse IL-2 and IFN-γ (eBioscience), according to the manufacturer's instructions. Capture antibodies were diluted at the recommended concentration in PBS and immobilized onto Immulon 4 HBX, flat-bottomed 96-well plates (Thermo Labsystems, VWR International, Mississauga, ON, Canada) overnight at 4 °C. Following immobilization, wells were washed once with wash buffer (0.5% PBS/Tween) and blocked using 200 µl of assay diluent (10% FBS in PBS) for 1-2 hours at room temperature. Samples and standards were then added, topped up to 100 µl per well and incubated for a further 2 hours at room temperature. Wells were washed three times prior to the addition of the detection antibodies. Following one hour incubation and three more washes, avidin-HRP was added at the recommended concentration and incubated for 20 minutes at room temperature. Plates were washed at least three times before addition of substrate; TMB (TetraMethylBenzidine, eBioscience). After sufficient color had developed, the reaction was stopped using 2 M H_2SO_4 and absorbance at 450 nm was measured on a SPECTRAmax PLUS^{384} plate reader (Molecular Devices) using 630 nm as a reference wavelength.

Cell Isolations. Splenocytes and lymph node cells (inguinal, axillary, brachial, and submandibular) from wild-type or IFN-γR1 -/- mice were harvested and processed to a single cell suspension by pressing against a steel mesh with a syringe plunger. CD4 and CD8 were separated using SpinSep separation system (StemCell, Vancouver, BC, Canada) for reconstitution MLC experiments according to the manufacturer's instructions. Purity of isolated cells was between 90-94%. The number of viable isolated CD4 and CD8 cells was added to MLC in 24-well plates according to their respective presence in splenocyte preparations from unstimulated mice.

Alternatively, CD4 and CD8 cells were separated from spleen and lymph nodes by positive selection using EasySep separation (StemCell, Vancouver, BC, Canada) according to the manufacturer's instructions. Purity of isolated cells was between 92-96%.

Flow Cytometry. Cells from various experiments were collected and washed in flow cytometry staining buffer (0.5% FBS, 0.5% EDTA, 0.05% sodium azide in PBS). $5x10^5$ cells were used per tube for flow cytometry analysis. Prior to immunolabelling, Fc receptors were blocked using anti-mouse FcγRIII/II antibody (eBioscience) for 5 minutes at room temperature, to decrease the amount of non-specific staining. Samples were stained with various combinations of directly conjugated antibodies: anti-CD3ε (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD44 (IM7), anti-CD62L (MEL14), anti-Ly6G (RB6-8C5), anti-F4/80 (BM8; eBioscience), and anti-CXCR3 (220803; R&D,

Minneapolis, MN), for 10-15 minutes at room temperature. Cells were washed with staining buffer, and fixed in PBS/1 % paraformaldehyde and stored at 4°C until analyzed.

Cells were analyzed using a FACScan or FACScalibur flow cytometer along with CellQuest software (BD Immunocytometry Systems, Mississauga, ON, Canada).

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR). RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer's instructions. Cells were resuspended in residual volume of media following removal of supernatant and TRIzol was added to the tubes at 1 ml per $2x10^7$ cells. Four μg of RNA were reverse transcribed in a 40 μL reaction containing 1X first strand buffer, 25 ng/μL random primers, 2 μM dNTPs, 80 μg/ml BSA, 8 mM DTT, 400 U M-MLV reverse transcriptase (Invitrogen) and 80 U RNasein RNase inhibitor (Promega, Madison, WI) at 37 °C for 60 minutes, and then heated to 95 °C for 5 minutes. Resulting cDNA was amplified and detected on an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems) using gene-specific primers and labeled probes (Table 2.1). Each 25 μl reaction consisted of 1 x TaqMan Universal PCR Master Mix (PE Applied Biosystems), forward and reverse primers, and TaqMan quantification probe, each at their optimized concentrations, and 50 ng of cDNA template. PCR amplification conditions were as follows: 50°C for two minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for one minute. All PCR reactions were performed in triplicate. Amplification plots were constructed using ABI PRISM 7700 Sequence Detection System software version 1.7 (PE Applied Biosystems). Threshold cycle numbers (Ct) were determined and transformed using the ddCt methods as described by the

manufacturer, using murine hypoxanthine phosphoribosyltransferase (mHPRT) as the internal reference. The following equation was used to express the relative amounts of mRNA: $2^{-[(Ct\ target\ gene_X - Ct\ HPRT_X)-(Ct\ target\ gene_{calibr} - Ct\ HPRT_{calibr})]}$ where 'calibr' refers to the values obtained from the unstimulated sample in the experiment.

Allogeneic tumor challenge. To generate allo-specific CTL we injected allogeneic P815 mastocytoma cells into wild-type, IFN-γ -/-, or IFN-γR1 -/- mice. Cells were collected from culture, washed and resuspended in sterile PBS. The indicated numbers of P815 cells, in a volume between 0.2-0.4 ml of sterile PBS, were injected into wild-type and IFN-γR1 -/- mice. After seven days, draining lymph node (axillary, in subcutaneous injections) and spleen (in subcutaneous and i.p. injections) were collected. Single cell suspensions were prepared in complete RPMI as described above. Lymph node and spleen cells were used in functional studies or characterized by flow cytometry.

Restimulation assays. P815 or T6SV (H-2^k) cells, and DBA/2 (H-2^d) or CBA (H-2^k) splenocytes were collected as described above. Cells were treated with mitomycin C at 25 μg/ml for 20 minutes at 37 °C. $2x10^5$ lymph node or spleen cells from P815-challenged mice were restimulated with mitomycin-treated P815 or T6SV cells and DBA/2 and CBA splenocytes. Supernatants were collected after 18 hours for determination of IFN-γ levels.

Recombinant cytokines and cytokine neutralizations. Recombinant IL-2 and IFN-γ (Peprotech, Rocky Hill, NJ) were dissolved in 0.5% BSA in filter sterilized PBS,

aliquoted, and kept at -70°C until used. IL-2 was added at a dose of 10 U/ml at 24 hours and 48 hours of MLC. IFN-γ was added at the indicated doses after 24 hours of MLC.

Anti-IL-2 antibody (JES6-1A12, eBioscience) was used to neutralize IL-2 in MLC at a dose of two μg/ml, added at 24 and 48 hours as indicated. IFN-γ was neutralized using the antibody XMG1.2 (eBioscience), at two μg/ml, at 24 and 48 hours of culture. For *in vivo* neutralization of IL-2, anti-IL-2 (JES6-1A12, eBioscience) with no azide and low endotoxin levels, was injected in the indicated IFN-γR1 -/- mice that had been challenged with P815 cells. Anti-IL-2 was injected i.p. 24 hours following subcutaneous P815 injection.

Statistical analysis. Statistical analyses were performed using SPSS software (SPSS Incorporated, Chicago, IL). Student's *t*-test was used in the analysis of most experiments unless otherwise indicated.

2.3 TABLE

Table 2.1: Gene-specific primer and probe sequences used in real time RT-PCR studies.

Primer/Probe	Sequence (5'-3')
Granzyme B fwd	CGATCAAGGATCAGCAGCCT
Granzyme B rev	CTTGCTGGGTCTTCTCCTGTTCT
Granzyme B probe	fam –TGCTGCTCACTGTGAAGGAAGTATAATAAATGTCACT
HPRT fwd	TGACACTGGTAAAACAATGCAAACT
HPRT rev	AACAAAGTCTGGCCTGTATCCAA
HPRT probe	vic –TTCACCAGCAAGCTTGCAACCTTAACC

2.4 REFERENCE LIST

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2 Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R. M., and Aguet, M. 1993. Immune response in mice that lack the interferon-gamma receptor. *Science* 259:1742-1745.

CHAPTER 3

EFFECTS OF IFN-γ ON CTL GENERATION IN MLC

(Parts of the data presented in this chapter have been published. Hidalgo, L.G., Urmson J. and Halloran P.F. 2005. IFN-γ decreases CTL generation by limiting IL-2 production: A feedback loop controlling effector cell production. American Journal of Transplantation. 5:651-61. Used with permission.)

3.1 INITIAL *IN VIVO* STUDIES IN WILD-TYPE AND IFN-γR1 -/- HOSTS.

Our general interest was in the roles played by IFN-γ in allograft rejection. We first sought to determine the reason for accelerated rejection of kidney allografts in hosts lacking IFN-γ (1). One possibility was that CTL responses in IFN-γ -/- hosts develop more vigorously, leading to excessive destruction of cells in the graft. The effect of IFN-γ on *in vivo* CTL responses was determined using 129/B6 F1 (H-2^b) or IFN-γR1 -/- hosts into which we injected allogeneic P815 cells ($5x10^6$ per mouse) in a volume of 0.2-0.4 ml of sterile PBS intraperitoneally (i.p.). CTL lytic activity was determined in the spleen after seven days. Single cell splenocyte suspensions depleted of RBCs were used as effectors against ^{51}Cr labeled P815 or RDM-4 (H-2^k) cells as targets. Three different effector:target ratios were used in a standard four hour ^{51}Cr release assay: 50:1, 10:1, and 2:1 (Figure 3a). CTL lytic activity was relatively weak in wild-type hosts compared to IFN-γR1 -/- hosts after seven days, even at the highest effector:target ratio used. Lytic activity was detectable at 10:1 effector:target ratios in IFN-γR1 -/- but not wild-type

hosts. At the highest effector:target ratio, IFN-γR1 -/- hosts showed approximately six times greater cytolytic activity than wild-type hosts (Figure 3a) ($p<0.0001$) and this trend was also observed at the 10:1 effector:target ratio. Lytic activity displayed by both wild-type and IFN-γR1 -/- hosts was allospecific, since killing of RDM-4 targets, which express H-2^k on their surface, remained below 5% at all effector:target ratios for both types of effectors (Figure 3a - i).

3.2 LYTIC ACTIVITY OF WILD-TYPE AND IFN-γR1 -/- EFFECTORS.

Having established that CTL generation is enhanced in IFN-γR1 -/- hosts, we investigated the mechanism. To better manipulate conditions and more easily assay responses, we studied CTL generation in response to allogeneic stimulation in mixed leukocyte reactions (MLC). Our first aim was to duplicate in a MLC our *in vivo* results obtained by injecting P815 cells into wild-type and IFN-γR1 -/- hosts. We utilized spleens from BALB/c mice and prepared single-cell suspensions, lysed RBCs, treated with mitomycin C (25 μg/ml) and used these as stimulators. Splenocytes from wild-type 129/B6 F1 mice or IFN-γR1 -/- hosts were used as responders. Responders and stimulators were mixed together at a concentration of $5x10^6$ cells/ml in complete RPMI and cytolytic activity measured by ^{51}Cr release assay after five days. The three effector:target ratios used to assess lytic activity in MLC were: 30:1, 10:1, and 3:1. Following five days of culture, IFN-γR1 -/- effectors displayed stronger lytic activity against P815 targets compared to wild-type responders (Figure 3b). On average killing of P815 targets by IFN-γR1 -/- effectors was twice of that observed with wild-type effectors at all effector:target ratios

tested ($p<0.01$). The killing was once again allospecific since killing of RDM-4 targets was minimal and equal between IFN-γR1 -/- and wild-type responders (Figure 3b - i).

3.3 ESTABLISHMENT OF IFN-γ -/- MLC. To elucidate the direct effects of IFN-γ on CTL generation in MLC we established a MLC where IFN-γ would be absent. We initially attempted to use BALB/c stimulators along with C57BL/6 IFN-γ -/- responders (GKO vs WT) and compared those to MLC composed of BALB/c stimulators and C57BL/6 responders (WT vs WT). Lytic activity was measured after five days of culture. The effector population generated in MLC containing IFN-γ -/- responders killed allogeneic targets better than effectors from wild-type responders but not nearly as well as the effector population from IFN-γR1 -/- responders (Figure 3c) ($p<0.005$ for MLC containing IFN-γ -/- responders compared to $p<0.0001$ for IFN-γR1 -/- responders). We hypothesized that a possible cause for this would be that, although BALB/c stimulators were treated with mitomycin C and therefore not able to proliferate, they may retain the ability to produce a certain amount of IFN-γ. To determine whether IFN-γ was present in MLC containing IFN-γ -/- responders but wild-type stimulators, we analyzed supernatant samples taken every 24 hours during the first four days of the MLC. We assayed IFN-γ levels in the supernatants of MLC having responders capable of producing IFN-γ (WT vs WT) and IFN-γ -/- responders (GKO vs WT) by ELISA (Figure 3d). The ELISA assay showed that IFN-γ was produced in MLC with IFN-γ -/- responders albeit at lower levels compared to those found in IFN-γ sufficient MLC, confirming our suspicion of IFN-γ production by stimulator cells.

To prepare a MLC completely deficient of IFN-γ, MLC were generated by combining C57BL/6 IFN-γ -/- responders together with BALB/c IFN-γ -/- stimulators. Lytic activity from IFN-γ deficient MLC (GKO vs GKO) was compared to that of IFN-γ sufficient MLC (WT vs WT) after five days against ^{51}Cr labeled P815 target cells (Figure 3c). Once IFN-γ was completely absent from the culture, the MLC produced lytic activity two to three times higher than IFN-γ sufficient MLC at all three effector:target ratios ($p<0.0001$ comparing % Lysis between IFN-γ deficient MLC and MLC and IFN-γ sufficient MLC). This was similar to that observed with IFN-γ R1 -/- responders previously with lysis of P815 target cells ($p=0.155$ comparing % Lysis between IFN-γ deficient MLC and MLC with IFN-γ R1 -/- responders). To demonstrate that the effects we observed were directly caused by IFN-γ we added recombinant IFN-γ (50 U/ml) to IFN-γ deficient MLC after 24 hours of culture (GKO + IFN). Exogenous IFN-γ neutralized the increased lytic activity resulting from the lack of IFN-γ (Figure 3c). These experiments helped to confirm that the effects we had observed in MLC with IFN-γR1 -/- responders were a direct effect of IFN-γ on the generation of lytic activity and also ruled out any developmental defects by IFN-γ -/- cells.

3.4 REGULATION OF IL-2 PRODUCTION BY IFN-γ. Having established that the generation of lytic activity is increased *in vivo* and in MLC through a direct effect of IFN-γ, we began searching for a possible mechanism. The cytokine IL-2 has potent activity in the generation of lytic activity (2) and was one of the parameters we decided to measure. We hypothesized that IFN-γ negatively regulated IL-2 levels in MLC and thus

negatively regulated the generation of lytic activity. IL-2 levels were determined in the supernatants of various MLC by taking samples every 24 hours during the first four days of culture and performing an ELISA assay. Supernatants from IFN-γ sufficient MLC (WT vs WT), IFN-γ deficient MLC (GKO vs GKO), and MLC with IFN-γR1 -/- responders (GRKO vs WT) were analyzed for IL-2 levels. The kinetics of IL-2 production was similar amongst the different MLC with IL-2 levels already detectable at 24 hours, increasing and peaking at 72 hours, followed by a decrease at 96 hours (Figure 3e). Although after 24 hours IL-2 levels were similar between all cultures tested, at 48 hours both IFN-γ deficient MLC and MLC with IFN-γR1 -/- responders showed approximately a two-fold increase in IL-2 levels which continued to increase to approximately three-fold at 72 hours when compared to IFN-γ sufficient MLC (Figure 3e) ($p<0.0001$ comparing IL-2 levels between IFN-γ deficient MLC and MLC with IFN-γR1 -/- responders to IFN-γ sufficient MLC after 72 hours). IL-2 levels in IFN-γ deficient MLC, like with lytic activity, were decreased to levels similar to those observed in IFN-γ sufficient MLC by the addition of recombinant IFN-γ (50 U/ml) at 24 hours of culture (GKO + IFN-γ). Similarly, at 72 hours IL-2 levels in IFN-γ sufficient MLC were increased by the addition of anti-IFN-γ (clone XMG1.2, two μg/ml) when added after 24 hours of culture ($p<0.0001$). Altogether these results demonstrate that IFN-γ decreases IL-2 levels in MLC and this may play a role in the enhanced generation of lytic activity observed in MLC where IFN-γ effects are absent.

To confirm the effect of IFN-γ on IL-2 levels in MLC, we performed a dose response curve for the addition of recombinant IFN-γ to IFN-γ deficient MLC. IL-2 levels were determined by ELISA after 72 hours of culture (the peak time point for this

cytokine). Recombinant mouse IFN-γ was added at five U/ml, 20 U/ml, and 100 U/ml after 24 hours of culture. IFN-γ decreased IL-2 levels at 72 hours in MLC in a dose dependent manner (Figure 3 f), confirming the negative effect of IFN-γ on IL-2 levels in MLC.

3.5 EFFECTS OF IL-2 ON THE GENERATION OF LYTIC ACTIVITY IN MLC. If the increased IL-2 levels present in IFN-γ deficient MLC and MLC with IFN-γR1 -/- responders are the cause of the increased lytic activity observed, altering IL-2 should impact lytic activity generated in MLC. To examine this hypothesis, we first decided to neutralize IL-2 in IFN-γ sufficient MLC (WT vs WT) and MLC with IFN-γR1 -/- responders by adding anti-mouse IL-2 (clone JES6-1A12, two μg/ml) at the start of the MLC (WT + αIL-2, and GRKO + αIL-2, respectively). The lytic activity generated after five days was compared to that of IFN-γ sufficient MLC (WT vs WT), MLC with IFN-γR1 -/- responders (GRKO vs WT), IFN-γ sufficient MLC and MLC with IFN-γR1 -/- responders treated with isotype control antibody (WT + isot, and GRKO + isot, respectively). Neutralizing IL-2 in MLC with IFN-γR1 -/- responders decreased lytic activity by approximately 80% at the three effector:target ratios assayed (Figure 3g - i) ($p<0.0001$), demonstrating the importance of IL-2 in the generation of lytic activity *in vitro*. Likewise, IL-2 neutralization in IFN-γ sufficient MLC also decreased the lytic activity of those effectors ($p<0.01$). The effect of the antibody neutralization was specific to IL-2, since the isotype treated cultures generated equal lytic activity to that observed in the untreated MLC. These experiments demonstrate that the generation of lytic activity in MLC is highly dependent on IL-2 levels in agreement with previous studies (2).

Furthermore, the data strongly suggests that the increased IL-2 levels present in IFN-γ deficient MLC and MLC with IFN-γR1 -/- responders are responsible for the increased lytic activity generated compared to IFN-γ sufficient MLC.

To determine whether the lytic activity enhancing effects of IL-2 can overcome the inhibitory effects of IFN-γ we decided to determine the effect of the addition of exogenous IL-2 to IFN-γ sufficient MLC. Recombinant mouse IL-2 was added to IFN-γ sufficient MLC at a dose of 10U/ml after 24 and 48 hours of culture (WT + IL-2) and lytic activity determined and compared to that of IFN-γ sufficient (WT vs WT) and IFN-γ deficient (GKO vs GKO) MLC (Figure 3g - ii). The addition of IL-2 to IFN-γ sufficient MLC increased the lytic activity generated by approximately 50-70%, approaching levels observed in IFN-γ deficient MLC. These experiments demonstrated two things: the first is further evidence of the ability of IL-2 to dictate the levels of lytic activity generated in MLC, and second, that IL-2 can increase lytic activity even in the presence of IFN-γ.

3.6 EFFECTS OF IL-2 ON IFN-γ PRODUCTION IN MLC. IFN-γ production is commonly used as a readout of effector T cell function during alloimmune responses (3). Given that we had shown that IL-2 increases the generation of lytic activity in MLC, we hypothesized that IL-2 would also increase IFN-γ levels. We determined IFN-γ levels in the supernatants of IFN-γ sufficient MLC (WT vs WT), IFN-γ sufficient MLC with exogenous IL-2 (WT + IL-2) as performed above, IFN-γ deficient MLC (GKO vs GKO), MLC with IFN-γR1 -/- responders (GRKO vs WT), and MLC with IFN-γR1 -/- responders treated with antibody to the IL-2 receptor α chain (CD25, clone PC61) added

at five μg/ml at the start of the culture (GRKO + αCD25) (Figure 3h). IFN-γ levels in samples taken every 24 hours from the MLC listed above were quantified by ELISA. The kinetics of IFN-γ levels in MLC supernatants followed a similar pattern as those for IL-2, with the exception that IFN-γ levels were not detectable at 24 hours of culture but present in high amounts at 48 hours, peaking at 72 hours, and decreasing by 96 hours. MLC with IFN-γR1 -/- responders contained higher levels of IFN-γ compared to IFN-γ sufficient MLC at all time points when IFN-γ levels were detectable (Figure 3h - i) ($p<0.01$). When CD25 was blocked in MLC with IFN-γR1 -/- responders, IFN-γ levels were decreased to levels even below those of IFN-γ sufficient MLC ($p<0.01$). This result suggests that IFN-γ levels in MLC may also be indicative of the number of alloreactive effectors present, since the results correlated with the effect of IL-2 neutralization in MLC on the generation of lytic activity (Figure 3g - i). In agreement with the experiments above, IFN-γ levels were also increased by the addition of exogenous IL-2 to IFN-γ sufficient MLC, compared to untreated IFN-γ sufficient MLC (Figure 3h - ii) ($p<0.001$). This supports the concept that IFN-γ levels are increased in proportion to the number of alloreactive effectors generated. The lack of IFN-γ detectable by ELISA in IFN-γ deficient MLC served to confirm that IFN-γ is absent in these MLC.

3.7 MECHANISM OF LYSIS BY EFFECTORS GENERATED IN THE PRESENCE OR ABSENCE OF IFN-γ IN MLC. The addition of IL-2 can increase the lytic activity of cultured T cells. CTL lytic activity requires the cytolytic molecules perforin and granzymes (4). The perforin/granzyme system is Ca^{2+}-dependent and is the most commonly used mechanism by which CTL lyse targets *in vitro*, but CTL are also

able to kill through the Ca^{2+}-independent Fas/FasL system (5). To determine the mechanism of killing and to demonstrate that IL-2 increases Ca^{2+}-dependent killing, we performed ^{51}Cr release assays using effectors generated after five days of MLC with and without pretreatment with EGTA/Mg^{2+} (two mM and five mM, respectively) for two hours prior to the assay (Figure 3i). We compared lytic activity of IFN-γ sufficient MLC (WT vs WT), IFN-γ sufficient MLC with exogenous IL-2 (WT + rIL-2), IFN-γ deficient MLC (GKO vs GKO), and IFN-γ deficient MLC with exogenous IL-2 (GKO + rIL-2) at an effector:target ratio of 30:1. As we had previously shown, the addition of IL-2 to IFN-γ sufficient MLC increased the lytic activity by approximately three-fold ($p<0.0005$). The same effect was observed with the addition of IL-2 to IFN-γ deficient MLC, which increased lytic activity from 78% to 95% lysis ($p<0.005$). Most importantly, the lytic activity of all effectors assayed was completely abolished by pretreatment with EGTA/Mg^{2+} demonstrating that the increased lytic activity observed in the absence of IFN-γ or by the addition of recombinant mouse IL-2 was due to Ca2+-dependent lysis and thus perforin- and granzyme-mediated.

3.8 EFFECTS OF IFN-γ AND IL-2 ON GRANZYME B mRNA LEVELS IN MLC. Differentiation of CD8 T cells into CTL involves the increased expression of many effector molecules including granzyme B (4). The experiments above showed that the amount of Ca2+-dependent lysis increased in the absence of IFN-γ and also by the addition of IL-2. This strongly suggests that there would be an increase in the number of cells expressing cytolytic effector molecules such as perforin or granzyme B. We hypothesized that granzyme B mRNA levels would be higher in IFN-γ sufficient MLC

with exogenous IL-2 and IFN-γ deficient MLC compared to IFN-γ sufficient MLC. To assess mRNA levels we performed MLC in 25 cm^3 flasks with 10 ml of responders and 10 ml of stimulators each at $5x10^6$ cells/ml. After four days of culture, live cells were collected and total RNA was extracted. cDNA was obtained by using reverse transcription along with random primers and the amounts of granzyme B mRNA steady state levels were determined by real-time PCR using granzyme B specific forward and reverse primers, and FAM-conjugated specific probe. Freshly isolated splenocytes from C57BL/6 (unstim WT) and IFN-γ -/- C57BL/6 mice (unstim GKO) were used as negative controls. Both of these showed low yet equal levels of granzyme B mRNA, as would be expected from a splenocyte population from unstimulated mice (Figure 3j). Also, this demonstrates that spleen cells from unstimulated IFN-γ -/- mice have equal levels of granzyme B compared to wild-type mice indicative of equal levels of activated CTL in the basal state.

Granzyme B levels increased nearly 100-fold in IFN-γ sufficient MLC after 96 hours of culture (96hrs WT vs WT) when compared to freshly isolated wild-type splenocytes. This is in agreement with an increased population of effector CTL by 96 hours compared to unstimulated splenocytes. Examination of IFN-γ sufficient MLC with exogenous IL-2 (96hrs WT-IL-2) and IFN-γ deficient MLC (96hrs GKO vs GKO) showed a higher amount of granzyme B expression when compared to unstimulated splenocytes and even when compared to IFN-γ sufficient MLC (Figure 3j). These results confirm our hypothesis and show that exogenous IL-2 and the absence of IFN-γ lead to higher levels of granzyme B mRNA, suggestive of an increased number of effector CTL generated in these MLC.

3.9 EFFECT OF IFN-γ AND IL-2 ON GENERATION OF ACTIVATED CD8 T CELLS IN MLC. We sought to confirm the presence of increased numbers of effector CTL brought about by the absence of IFN-γ or the addition of IL-2 as was strongly suggested by the increased lytic activity and expression of granzyme B in MLC. Due to the lack of reagents for intracellular staining for mouse perforin or granzyme B at the time of experiments, we chose to determine the activation state of CD8 T cells in MLC after four days of culture. Expression of the activation marker CD25 on CD8 T cells was used in these studies to determine the number of activated CD8 T cells. Activation state of CD8 T cells was determined in IFN-γ sufficient MLC (WT vs WT), IFN-γ sufficient MLC with exogenous IL-2 (WT-IL-2), IFN-γ deficient MLC (GKO vs GKO), IFN-γ deficient MLC with exogenous IFN-γ (GKO + IFN-γ), and MLC with IFN-γR1 -/- responders (GRKO vs WT). Results are shown as percent of T cells that express CD8 and CD25 (Figure 3k). These experiments showed that the percent of activated CD8 T cells in MLC after four days was highest in IFN-γ deficient MLC, MLC with IFN-γR1 -/- responders, and in IFN-γ sufficient MLC with exogenous IL-2, in agreement with the results where lytic activity and granzyme B mRNA levels were examined. Once again, similar to lytic activity results, the addition of recombinant IFN-γ to IFN-γ -/- MLC decreased the percent of activated CD8 T cells to levels comparable to those of IFN-γ sufficient MLC. These results are representative of five separate experiments. Altogether these experiments corroborate our previous findings and strongly suggest that the reason for the increased lytic activity in MLC when IFN-γ effects are disrupted is due

to the presence of an increased population of activated CD8 T cells (presumably effector CTL) resulting from increased levels of IL-2.

The increased percent of activated CD8 T cells in IFN-γ deficiency may be the result of increased generation, but can also be affected by the effect of IFN-γ on apoptosis in these cells. Since IFN-γ increases CD4 T cell apoptosis under some conditions (6), we decided to determine the percent of apoptotic CD8 cells in MLC with and without IFN-γ. Apoptotic CD8 cells were detected by flow cytometry after 96 hours of culture by using propidium iodide and Annexin V staining along with CD8 surface staining. We did not find differences in the percent of apoptotic (Annexin V+, Propidium iodide-) CD8 cells when we compared IFN-γ sufficient (WT vs WT), IFN-γ deficient (GKO vs GKO), and IFN-γ deficient plus recombinant IFN-γ (GKO + IFN) MLC (Table 3.1). Results shown are percent of CD8 cells and are representative of two independent experiments. Our data are in agreement with previous work showing that alloantigen-induced T lymphocyte apoptosis is not altered by IFN-γ (7). This suggests that the increased percent of activated CD8 T cells present in IFN-γ deficient MLC is a result of increased generation and rules out a role for IFN-γ in CD8 T cell apoptosis.

3.10 DETERMINING THE SOURCE OF IL-2 IN MLC. CD4 T cells are the primary producers of IL-2 in alloimmune responses, although CD8 T cells are also capable of producing IL-2 in smaller amounts (8). We have shown that IL-2 levels are increased in MLC deficient in IFN-γ effects. We hypothesized that this was a result of increased IL-2 production by CD4 T cells in these MLC. To determine the cell population responsible for the increased IL-2 levels, we performed IC staining for IL-2 in

cells from IFN-γ sufficient and IFN-γ deficient MLC. MLC were prepared as outlined above but performed in 96-well plates with 100 µl of stimulator cells along with 100 µl of responder cells for a total of $1x10^6$ cells/well. Since peak IL-2 levels were detected at 72 hours of culture, we performed the IC staining assay at that time point. We added monensin to the cultures according to the manufacturer's instructions after 66 hours of culture. After six hours of incubation with monensin, cells were collected, labeled for cell surface markers, washed, fixed and permeabilized prior to adding fluorescently labeled anti-mouse IL-2. Flow cytometry results showed that approximately 8% of CD4 T cells from IFN-γ sufficient MLC (WT vs WT) expressed IL-2 while 16% of CD4 T cells from IFN-γ deficient MLC (GKO vs GKO) expressed IL-2 (Figure 3l). This assay is representative of two separate experiments each with three samples per group. These results suggest that IFN-γ acts to limit the percent of IL-2 producing CD4 T cells.

IL-2 IC staining for the CD8 T cell populations showed that very little IL-2 was produced by these cells (Figure 3m), in agreement with previous findings showing that CD4 T cells are the primary producers of IL-2.

3.11 EARLY EFFECTS OF IFN-γ ON PROLIFERATION IN MLC. One of the most potent effects of IFN-γ is its anti-proliferative effect on numerous cell types (9). We thought that this may be the underlying reason for its limiting effects on IL-2 production. Thus, the following scenario could explain our findings: in the presence of IFN-γ the proliferation of all cells in MLC is hindered, including the responding CD4 T cell population responsible for the majority of the IL-2 produced, leading to decreased levels of IL-2 in the supernatant. For this scenario to be true there would have to be

increased proliferation in IFN-γ deficient MLC compared to IFN-γ sufficient MLC at 48 hours of culture: (This is the time at which differences in IL-2 levels were evident between IFN-γ sufficient and IFN-γ deficient MLC (Figure 3e)). To assess proliferation MLC were again performed in 96-well plates as outlined above. We used the MTT assay to determine proliferation of the cultures. In this assay MTT is added to the cultures (five μg/ml of media) and incubated at 37 °C for four hours. Thus we added MTT to two separate sets of MLCs, to one MTT was added after 44 hours and to the other after 92 hours. In each case the MTT was left for a further four hours before determining the amount of proliferation.

Proliferation was determined in IFN-γ sufficient MLC untreated (WT vs WT) or treated with IFN-γ neutralizing antibody (two μg/ml at 24 hours) (WT + XMG), and in IFN-γ deficient MLC untreated (GKO vs GKO) or treated with recombinant mouse IFN-γ (50 U/ml at 24 hours) (GKO + IFN). After 48 hours IFN-γ deficient MLC already showed increased proliferation compared to IFN-γ sufficient MLC (Figure 3n - i) ($p<0.03$). Neutralizing IFN-γ in IFN-γ sufficient MLC seemed to have a small effect in proliferation but this was not as great as the increase observed in IFN-γ deficient MLC. The addition of recombinant IFN-γ to IFN-γ deficient MLC also showed a noticeable decrease in proliferation at this early time point. This assay is representative of two separate experiments. These early effects on proliferation support the idea that the anti-proliferative effects of IFN-γ are responsible for the limited IL-2 production in MLC.

The anti-proliferative effects of IFN-γ are even more evident at later time points. When the MTT assay was performed at 96 hours of culture both IFN-γ deficient MLC and IFN-γ sufficient MLC treated with IFN-γ neutralizing antibody showed a striking

increase in proliferation compared to untreated IFN-γ sufficient MLC (Figure 3n - ii). The antibody neutralization of IFN-γ did not increase proliferation to the same extent as the complete absence of IFN-γ, probably because not all of the IFN-γ was neutralized, but did serve as a useful proof of concept. The addition of recombinant IFN-γ on the other hand decreased the proliferation of IFN-γ deficient MLC, and neutralization of IFN-γ increased proliferation of IFN-γ sufficient MLC, again demonstrating that this proliferation is a direct effect of IFN-γ. This assay is representative of three independent experiments.

3.12 IFN-γ ACTS ON CD4 T CELLS TO LIMIT CTL GENERATION. We have shown that CD4 T cells from IFN-γ deficient MLC produce greater amounts of IL-2 compared to CD4 T cells from IFN-γ sufficient MLC (Figures 3e and 3l), suggesting that IFN-γ limits CTL generation by an action on CD4 T cells. Although we have also shown that IL-2 mediates the increase of CTL generation, it was important to rule any indirect effects that IFN-γ may have by acting on CD8 T cells. To address this question we isolated CD4 and CD8 cells from lymph nodes and spleens of C57BL/6 and C57BL/6 IFN-γR1 -/- mice and added these in various combinations to BALB/c stimulator cells. MLC were conducted as before but in 24-well plates. CD4 and CD8 cells were added to simulate their respective abundance in splenocyte preparations from unstimulated mice: 25% of total cells ($2.2x10^6$ per well) were CD4 and 15% of cells ($1.15x10^6$ per well) were CD8. Lytic activity was determined in MLC containing wild-type CD4 and CD8 cells (WT CD4-WT CD8), wild-type CD4 and IFN-γR1 -/- CD8 (WT CD4-GRKO CD8), IFN-γR1 -/- CD4 and wild-type CD8 (GRKO CD4-WT CD8), and IFN-γR1 -/- CD4 and

CD8 (GRKO CD4-GRKO CD8) (Figure 3o). After five days of culture only the MLCs that contained IFN-γR1 -/- CD4 cells showed increased lytic activity against P815 targets at a 30:1 effector to target ratio, compared to MLCs with wild-type CD4 and CD8 cells. Surprisingly, the MLC containing IFN-γR1 -/- CD8 cells displayed moderately decreased lytic activity compared to MLCs with wild-type CD4 and CD8 cells. This experiment is representative of two separate experiments. The increase in lytic activity between MLCs with IFN-γR1 -/- CD4 and CD8 cells and those with wild-type CD4 and CD8 cells was only approximately 50% compared to the two- to three-fold increases observed when whole splenocytes were used in the MLC. This may highlight a role for accessory cells present in splenocyte populations in increasing CTL generation in MLC in the absence of IFN-γ effects. Despite the differences described, our data demonstrates that it is the effect of IFN-γ on CD4 T cells that limits CTL generation.

3.13 ENHANCED IL-2 PRODUCTION BY IFN-γR1 -/- CD4 T CELLS. Having demonstrated that the inability of CD4 T cells in a MLC to respond to IFN-γ was critical to increasing CTL lytic activity, we sought to demonstrate that CD4 cells in these cultures also produce higher levels of IL-2. To demonstrate increased IL-2 production by IFN-γR1 -/- CD4 T cells, we performed IC staining for IL-2 as above. The histograms show IL-2 production within the CD4 T cell gate (ie. CD3+4+) comparing wild-type CD4/IFN-γR1 -/- CD8 MLC with IFN-γR1 -/- CD4/wild-type CD8 MLC (Figure 3p). IL-2 was produced by a larger population of CD4 T cells when they were unable to respond to IFN-γ (GRKO CD4) than when they could respond (WT CD4) (35% vs 21%, respectively). The assay results are representative of two separate experiments. This

further confirmed that when IFN-γ is absent, CD4 T cells proliferate more than when IFN-γ is present. This experiment also demonstrates that the ability of the responding CD8 T cell population to receive IFN-γ does not affect the enhanced expansion of responding CD4 T cells.

3.14 TABLE.

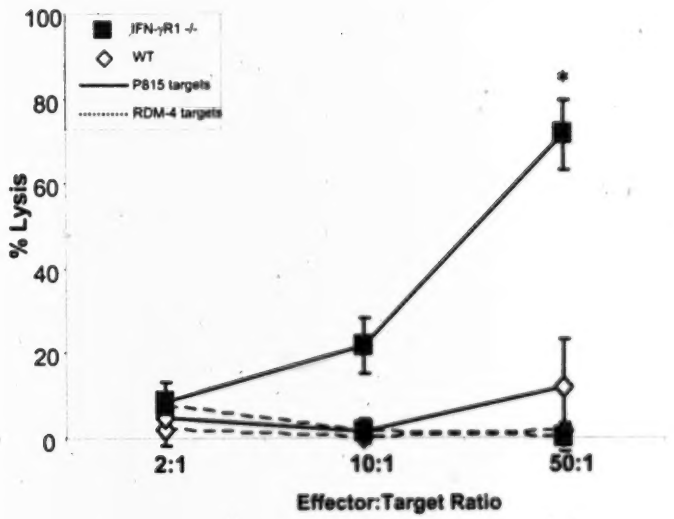
Table 3.1. Quantitation of apoptosis of CD8+ T cells after 96 hours of MLC in the presence and absence of IFN-γ. Data represents two independent experiments.

MLC Designation	% CD8+/Ann V+/PI-
WT vs WT	8.01
GKO vs GKO	8.75
GKO + IFN-γ	8.26

3.15 FIGURES

Figure 3 a. i) Generation of lytic activity in wild-type (WT) and IFN-γR1 -/- mice in response to allogeneic tumor challenge. P815 cells (H-2d) (5×10^6) were injected into WT and IFN-γR1 -/- mice i.p. and lytic activity of splenocytes determined after seven days. Results mean lysis ± SD. N = 3 samples per point and 3 mice per group. (* denotes significantly increased lytic activity compared to WT at 50:1 Effector:Target Ratio, $p<0.0001$). ii) Lytic units were calculated for the experiment above. One lytic unit was defined as the number of effector cells required to achieve 20% lysis of P815 targets. Data shown in i) and ii) are representative of one of five experiments.

i)


IFN-γR1 -/-
WT
P815 targets
RDM-4 targets
100
80
60
40
20
0
% Lysis
*
2:1
10:1
50:1
Effector:Target Ratio

ii)

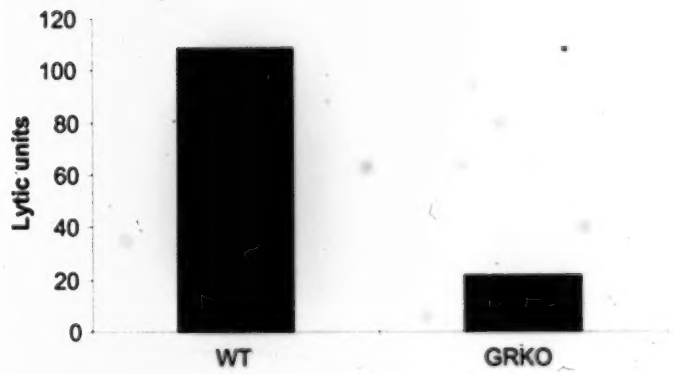
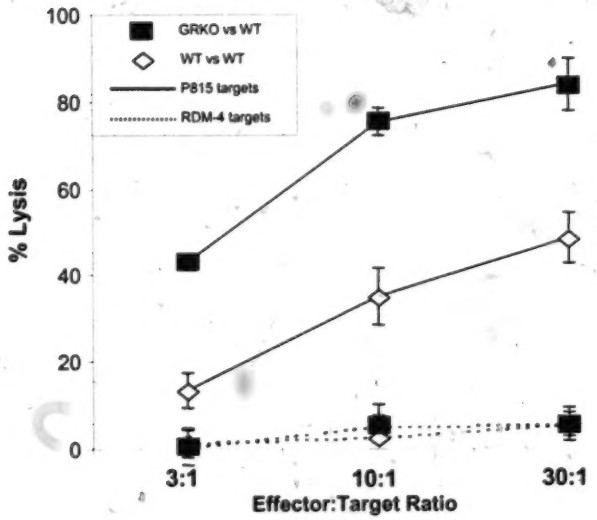
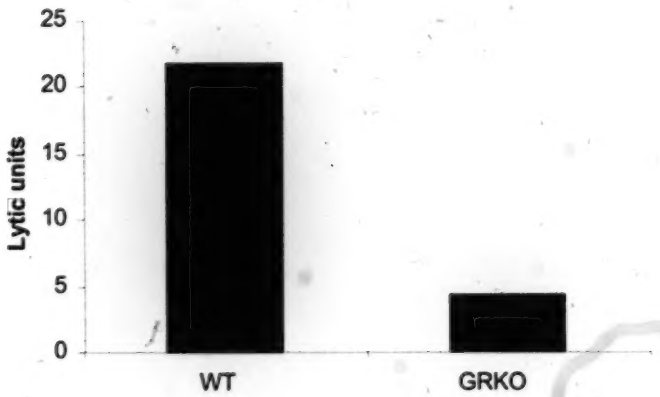

120
100
80
60
40
20
0
Lytic units
WT
GRKO

Figure 3 b. i) Effects of IFN-γR1 expression on responder population in MLC. 129/B6 F1 or IFN-γR1 -/- spleen and lymph node cells were stimulated with mitomycin C-treated BALB/c splenocytes. Lytic activity was determined after five days against P815 and RDM-4 targets. Results represent mean lysis ± SD of triplicate samples. Each experiment included 3 separate MLC, each composed of pooled stimulators from 3 mice and pooled responders from 3 mice. ($p<0.01$ at all Effector:Target Ratios using P815 targets). ii) Lytic units were calculated for the experiment above. One lytic unit was defined as the number of effector cells required to achieve 50% lysis of P815 targets. Data shown in i) and ii) are representative of one of five experiments.

i)


GRKO vs WT
WT vs WT
P815 targets
RDM-4 targets
% Lysis
100
80
60
40
20
0
3:1
10:1
30:1
Effector:Target Ratio

ii)


Lytic units
25
20
15
10
5
0
WT
GRKO

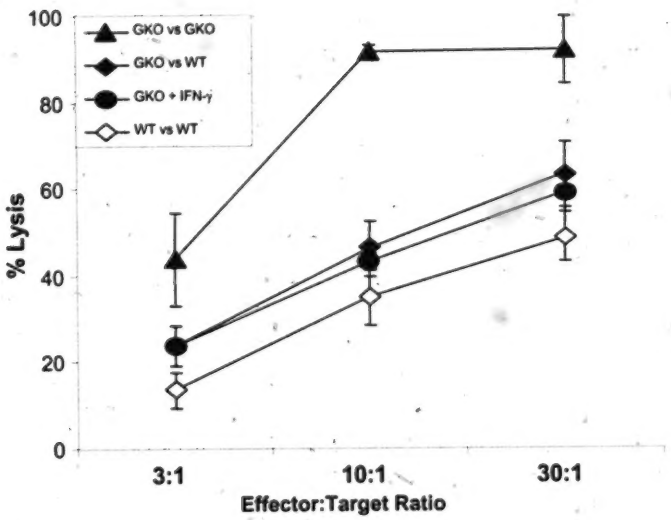


Figure 3 c. Limitation of lytic activity by IFN-γ in MLC. C57BL/6 or IFN-γ -/- spleen and lymph node cells were stimulated with mitomycin C-treated BALB/c or IFN-γ -/- splenocytes. Lytic activity was determined after five days against P815 targets. Results represent mean lysis ± SD of triplicate samples. Each experiment included 3 separate MLC, each composed of pooled stimulators from 3 mice and pooled responders from 3 mice. (p<0.0001 for GKO vs GKO, p<0.005 for GKO vs WT when compared to WT vs WT % Lysis at 30:1 Effector:Target Ratio). Data shown is representative of one of five experiments.

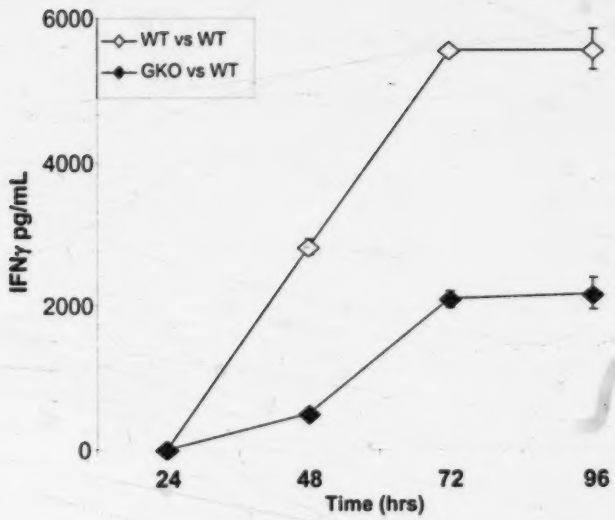


Figure 3 d. IFN-γ levels in supernatants of IFN-γ-competent MLC or MLC with IFN-γ -/- responders. MLC supernatants were collected every 24 hours and levels of IFN-γ were detected by ELISA. Results represent mean IFN-γ values ± SD. Each experiment included 3 separate MLC, each composed of pooled stimulators from 3 mice and pooled responders from 3 mice. Data shown is representative of one of five experiments.

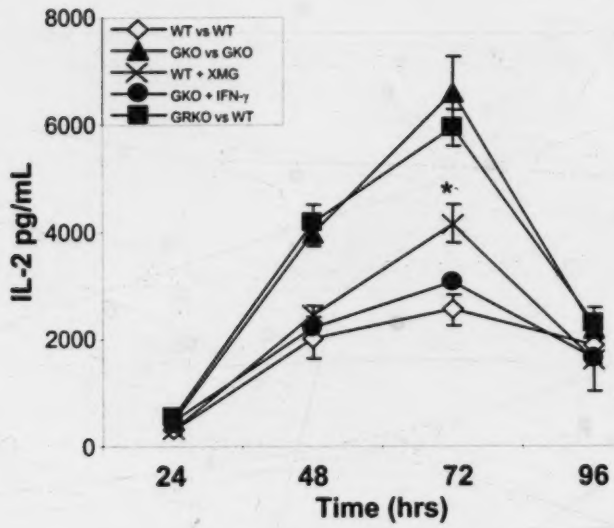


Figure 3 e. IL-2 levels in supernatants of IFN-γ-competent or IFN-γ-deficient MLC. MLC supernatants were collected every 24 hours and IL-2 levels were detected by ELISA. IFN-γ was added to IFN-γ deficient MLC (GKO + IFN-γ) (50 U/mL, final concentration), after 24 hours of culture. IFN-γ was neutralized in IFN-γ sufficient MLC (WT + XMG) by adding XMG1.2 antibody (2.0 μg/mL) after 24 and 48 hours of culture. Results represent mean IL-2 values ± SD of triplicate samples. Each experiment included 3 separate MLC, each composed of pooled stimulators from 3 mice and pooled responders from 3 mice. (* denotes significantly increased IL-2 levels compared to WT vs WT at 72 hours, $p<0.05$, $p<0.0001$ for GKO vs GKO and GRKO vs WT compared to WT vs WT at 72 hours).

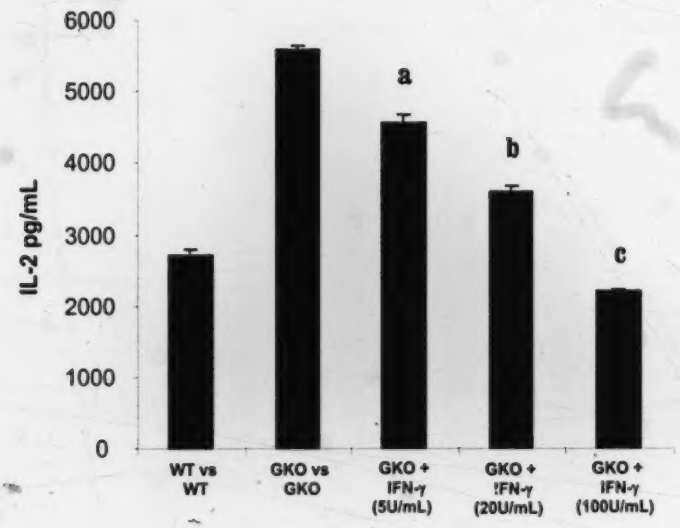
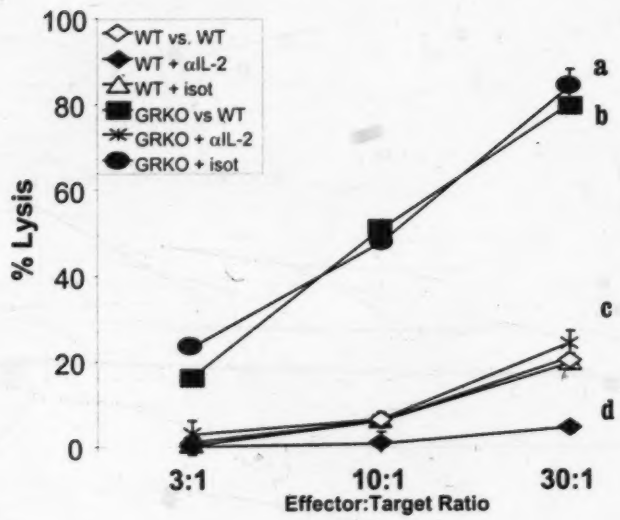


Figure 3 f. Dose response of IFN-γ effects on IL-2 levels in MLC supernatants. MLC supernatants were collected after 72 hours of culture and IL-2 levels were determined by ELISA. IFN-γ was added to IFN-γ deficient MLC (GKO vs GKO) at various concentrations after 24 hours of culture (GKO + IFN-γ). Results are representative of two independent experiments. Assays were performed in triplicate. Results represent mean IL-2 values ± SD. (**a** - denotes significantly decreased IL-2 levels $p<0.005$, **b** - $p<0.0002$, **c** - $p<0.0001$ when compared to GKO vs GKO).

Figure 3 g. IL-2 promotes, and IFN-γ limits CTL activity in MLC. CTL activity was determined by ^{51}Cr release assay on the fifth day of MLC using P815 target cells. All assays were performed in triplicate. i) CTL activity of IFN-γ sufficient MLC (WT vs WT) and MLC with IFN-γR1 -/- responders (GRKO vs WT) with anti-IL-2 (-αIL-2) or isotype control (-isot). Anti-IL-2 or isotype antibody was added to cultures at a dose of 2.0 μg/ml at 24 and 48 hours of culture. (a,b - denotes significantly higher % Lysis, $p<0.005$, c – denotes significantly decreased % Lysis, $p<0.005$ when compared to GRKO vs WT, d - denotes significantly decreased % Lysis, $p<0.005$ when compared to GRKO vs WT). ii) CTL activity of IFN-γ sufficient MLC, IFN-γ sufficient MLC plus recombinant IL-2 (WT-IL-2), and IFN-γ deficient MLC. IL-2 was added to WT-IL-2 cultures (10 U/mL, final concentration) at 24 and 48 hours. Means for one experiment ± SD are shown and are representative of three independent experiments for each. (a,b - denotes significantly higher % Lysis, $p<0.01$ when compared to WT vs WT).

i)


WT vs. WT
WT + αIL-2
WT + isot
GRKO vs WT
GRKO + αIL-2
GRKO + isot
% Lysis
100
80
60
40
20
0
a
b
c
d
3:1
10:1
30:1
Effector:Target Ratio

ii)

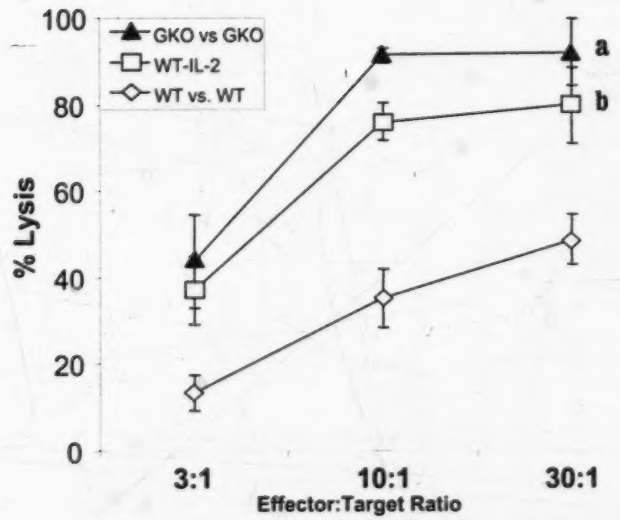
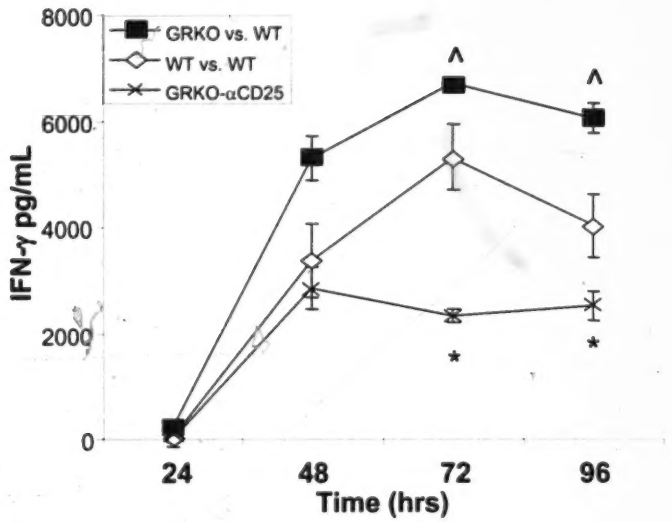
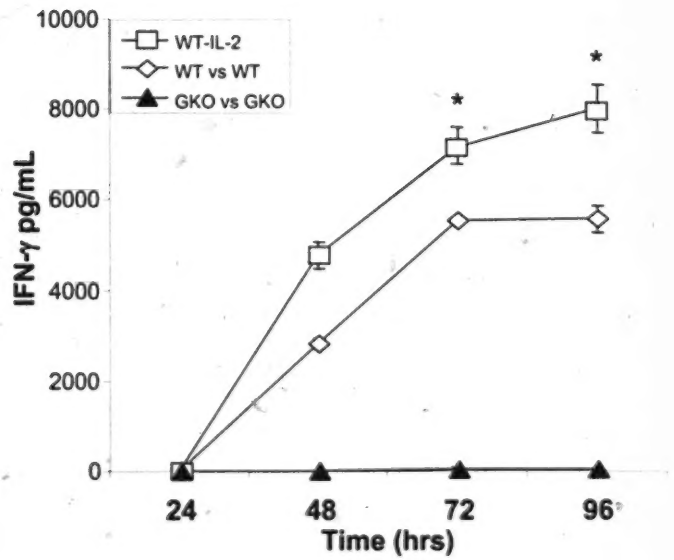

GKO vs GKO
WT-IL-2
WT vs. WT
% Lysis
100
80
60
40
20
0
a
b
3:1
10:1
30:1
Effector:Target Ratio

Figure 3 h. IFN-γ levels correlate lytic activity in MLC. MLC supernatants were collected every 24 hours and IFN-γ levels were detected by ELISA All assays were performed in triplicate. i) IFN-γ levels in IFN-γ sufficient MLC (WT vs WT), MLC with IFN-γR1 -/- responders (GRKO vs WT), and MLC with IFN-γR1 -/- responders treated with anti-CD25 (GRKO-αCD25). Anti-CD25 antibody was added to cultures at a dose of 5.0 μg/ml at the start of the culture. (^ - denotes significantly higher IFN-γ levels at 72 and 96 hours, $p<0.01$ when compared to WT vs WT, * - denotes significantly lower IFN-γ levels at 72 and 96 hours, $p<0.01$ when compared to WT vs WT). ii) IFN-γ levels in IFN-γ sufficient MLC, IFN-γ sufficient MLC plus recombinant IL-2 (WT-IL-2), and IFN-γ deficient MLC (GKO vs GKO). IL-2 was added to WT-IL-2 cultures (10 U/mL, final concentration) at 24 and 48 hours. Means for one experiment ± SD are shown (* - denotes significantly higher IFN-γ levels at 72 and 96 hours, $p<0.001$ when compared to WT vs WT). Data shown for A and B are representative of three independent experiments for each.

i)


GRKO vs. WT
WT vs. WT
GRKO-αCD25
IFN-γ pg/mL
8000
6000
4000
2000
0
24
48
72
96
Time (hrs)
^
^
*
*

ii)


WT-IL-2
WT vs WT
GKO vs GKO
IFN-γ pg/mL
10000
8000
6000
4000
2000
0
24
48
72
96
Time (hrs)
*
*
*

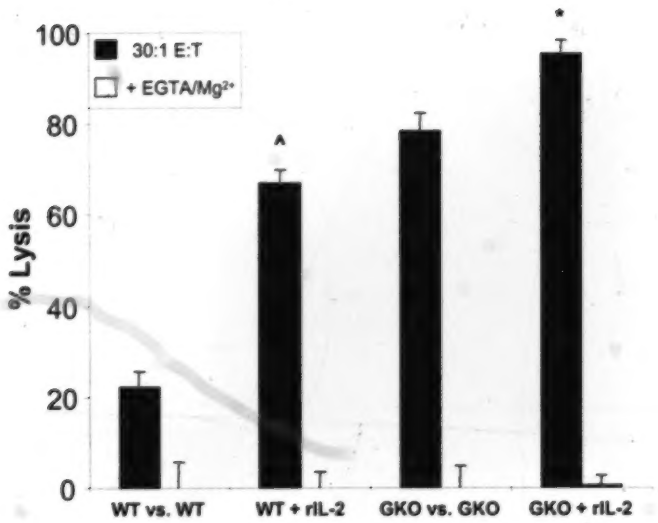


Figure 3 i. Effect of exogenous Ca^{2+} on CTL lysis of P815 targets. Lytic activity of IFN-γ sufficient MLC (WT vs WT) and IFN-γ deficient MLC (GKO vs GKO), alone or plus recombinant IL-2 (+ rIL-2), was assessed at an effector:target ratio of 30:1 (dark bars) and compared to the same effectors preincubated in 2 mM EGTA/5 mM $MgCl_2$ for 2 hours prior to assay (white bars). IL-2 was added to cultures as above. Means for one experiment ± SD are shown (assays performed in triplicate). (^ - denotes significantly higher % Lysis, $p<0.0005$ when compared to WT vs WT, * - denotes significantly higher % Lysis, $p<0.005$ when compared to GKO vs GKO). Data shown is representative of two independent experiments.

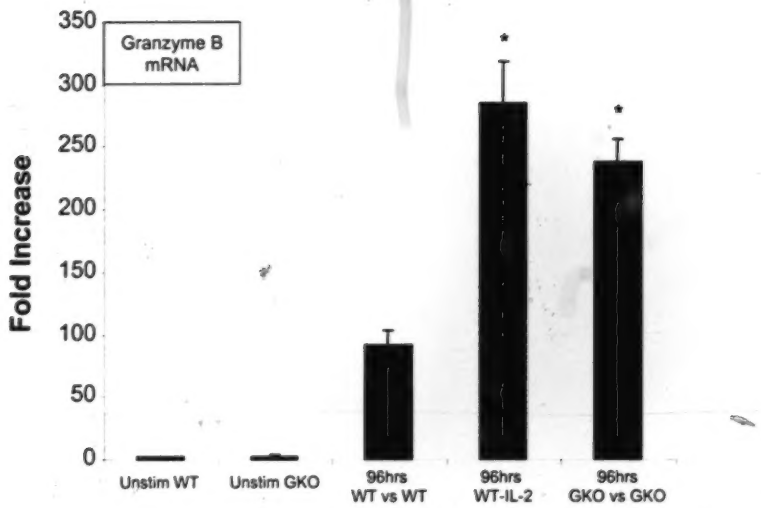


Figure 3 j. Granzyme B expression in MLC. mRNA levels for granzyme B were determined in RNA extracted from freshly isolated (unstim) wild-type (WT) or IFN-γ -/- (GKO) splenocytes, IFN-γ sufficient MLC (WT vs WT), IFN-γ sufficient MLC plus recombinant IL-2 (WT-IL-2), and IFN-γ deficient MLC (GKO vs GKO) cells after 96 hours of culture. Granzyme B mRNA levels were measured by real-time RT-PCR using (unstim) WT sample as the reference. Means for two experiment ± SD are shown (assays performed in triplicate) (* - denotes significantly higher Granzyme B mRNA increase, $p<0.0001$ when compared to 96h WT vs WT).

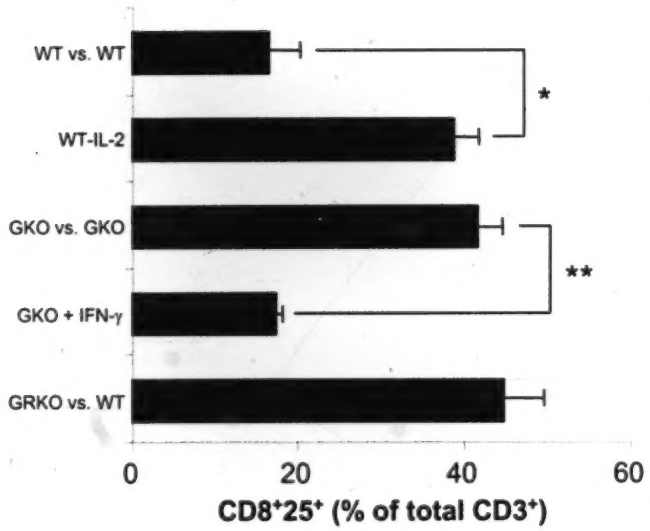


Figure 3 k. Opposite effects of IFN-γ and IL-2 on the generation of cells bearing markers of CTL effectors. Percent CD8+CD25+ T cells present in MLC was determined by flow cytometry. Means for one experiment ± SD are shown and are representative of five independent experiments (assays were performed in triplicate). Asterisks indicate significantly increased percent of CD3+8+25+ cells compared to WT vs WT (* $p<0.0005$) or reduced percent of CD3+8+25+ cells compared to GKO vs GKO (** $p<0.005$). IFN-γ and IL-2 were added to respective cultures as before.

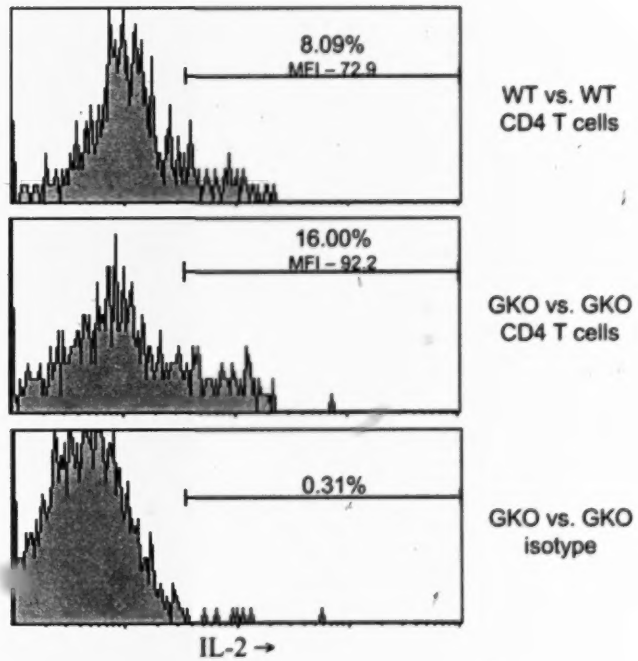


Figure 3 l. IL-2 production by IFN-γ sufficient and IFN-γ deficient CD4 T cells. Intracellular IL-2 was detected in CD4 T cells from IFN-γ sufficient (WT vs WT) and IFN-γ deficient (GKO vs GKO) MLC after 48 hours of culture. Percent of total T cells (CD3+) is shown. Graphs shown are representative of two independent experiments.

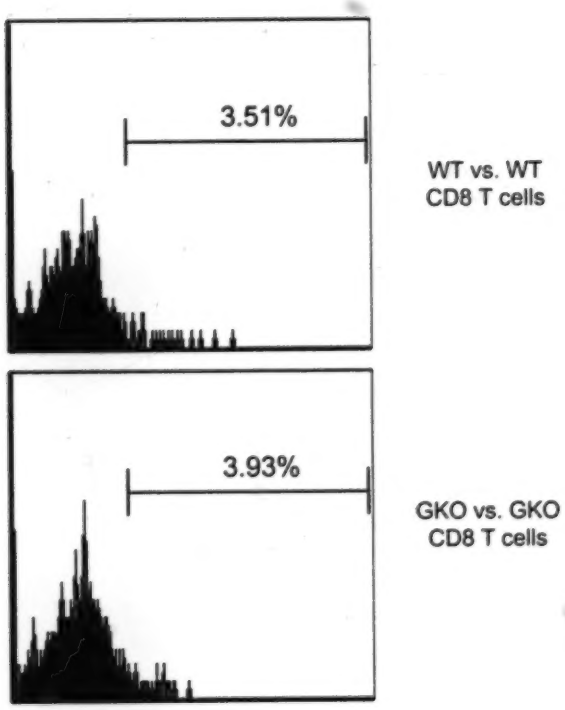


Figure 3 m. IL-2 production by IFN-γ sufficient and IFN-γ deficient CD8 T cells. Intracellular IL-2 was detected in CD4 T cells from IFN-γ sufficient (WT vs WT) and IFN-γ deficient (GKO vs GKO) MLC after 48 hours of culture. Percent of total T cells (CD3+) is shown. Graphs shown are representative of two independent experiments.

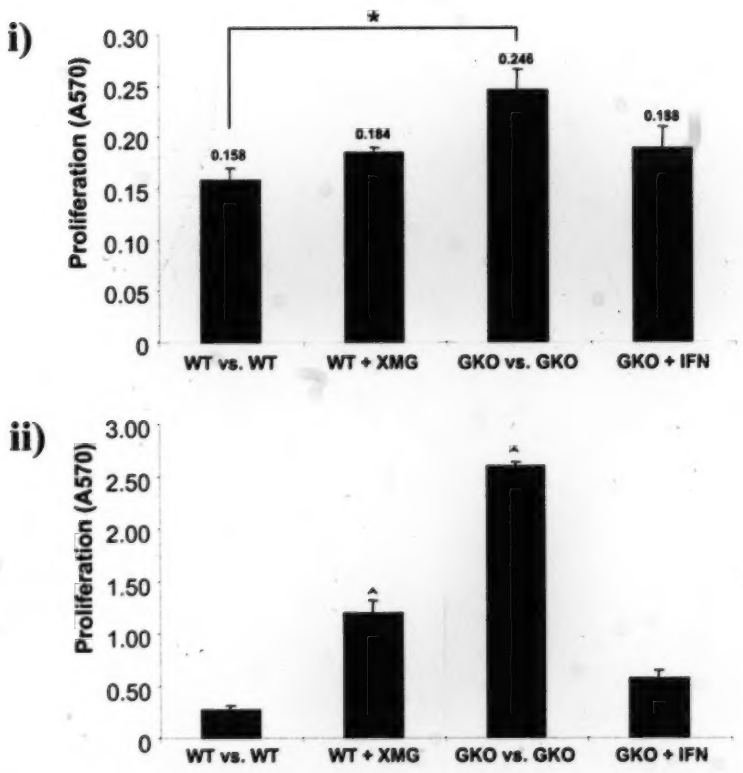


Figure 3 n. Proliferation rates in MLC. Cells were seeded in 96-well plates in triplicate. Proliferation was measured after 48 hours i) or 96 hours ii) of culture by the MTT assay in IFN-γ sufficient MLC (WT vs WT), IFN-γ sufficient MLC with IFN-γ neutralizing antibody, IFN-γ deficient MLC (GKO vs GKO), and IFN-γ deficient MLC plus recombinant mouse IFN-γ (GKO + IFN). Addition of rIFN-γ and XMG1.2 were performed as mentioned above. Means for one experiment ± SD are shown and are representative of two (i) or three (ii) independent experiments. (* denotes significantly increased proliferation compared to WT vs WT, $p<0.03$, ^ denotes significantly increased proliferation compared to WT vs WT, $p<0.0001$).

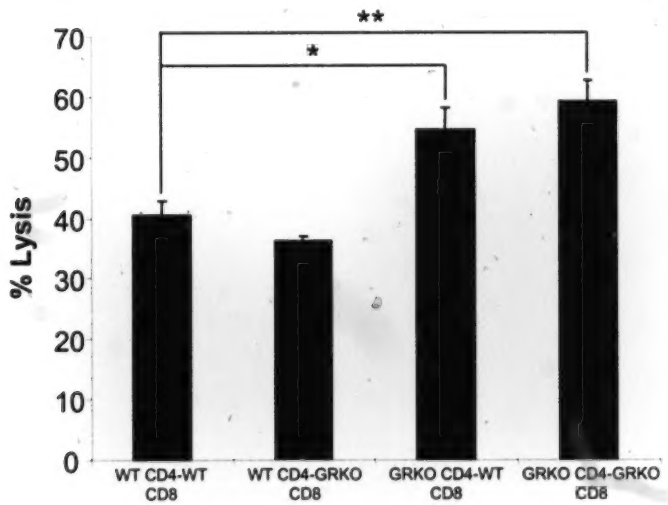


Figure 3 o. Inability of CD4 T cells to receive IFN-γ leads to increased lytic activity. CTL activity was determined as above. In MLC containing wild-type CD4 and CD8 cells (WTCD4-WTCD8), wild-type CD4 and IFN-γR1 -/- CD8 cells (WTCD4-GRKOCD8), IFN-γR1 -/- CD4 and wild-type CD8 cells (GRKOCD4-WTCD8), and IFN-γR1 -/- CD4 and CD8 cells (GRKOCD4-GRKOCD8). CTL activity was determined on the fifth day of MLC. (* denotes significantly increased % lysis compared to WTCD4 - WTCD8, $p<0.02$, ** denotes significantly increased % lysis compared to WTCD4 - WTCD8, $p<0.004$)

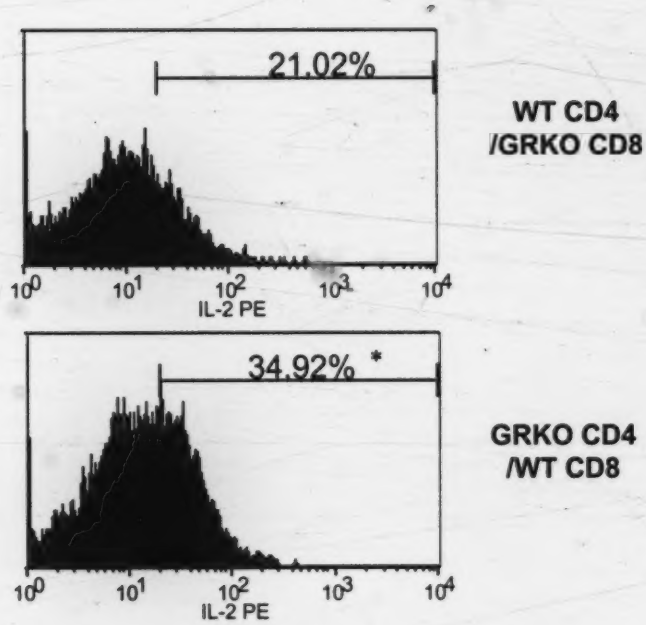


Figure 3 p. Inability of CD4 T cells to receive IFN-γ leads to an increased population of IL-2 producing cells. Intracellular levels of IL-2 in reconstituted cultures after 48 hours of culture were determined by flow cytometry. IL-2 was detected in MLC containing wild-type CD4 and IFN-γR1 -/- CD8 cells (WTCD4/GRKOCD8) and IFN-γR1 -/- CD4 and wild-type CD8 cells (GRKOCD4/WTCD8) after 48 hours of culture. Graphs show percent of total T cells (* denotes increased % IL-2+ T cells between WTCD4+GRKOCD8 and GRKOCD4/WTCD8 for three separate MLC, $p<0.01$). Data is representative of two independent experiments. Each experiment consisted of isolated cells from three individual mice.

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CHAPTER 4

THE ROLE OF IRF-1 IN CTL GENERATION IN MLC

Many of the effects of IFN-γ are mediated through the transcription factor IRF-1. Although IRF-1 -/- mice are viable, they display decreased numbers of CD8 T cells which may be linked to the decreased MHC class I expression resulting from the low levels of LMP2 and TAP1 (1;2).

4.1 EFFECTS OF IRF-1 ON IL-2 PRODUCTION IN MLC. We investigated whether IRF-1 was involved in the regulation of CD4 T cell responses during MLC. Since the defect in IRF-1 -/- mice seems limited to MHC class I and CD8 T cells, we anticipated that they might be useful to answer questions about CD4 T cell function. Our hypothesis was that IRF-1 limits proliferation of CD4 T cells. Thus, IRF-1 -/- CD4 T cells would produce greater amounts of IL-2 in MLC. We performed MLC using C57BL/6 or C57BL/6 IRF-1 -/- splenocytes as responders and mitomycin C-treated BALB/c splenocytes as stimulators using the same conditions as before. Culture supernatants were collected every 24 hours during the first 4 days of the cultures and IL-2 levels were determined by ELISA.

IL-2 levels in the MLC containing IRF-1 -/- responders followed a nearly identical pattern to that of IFN-γ deficient MLC or MLC with IFN-γR1 -/- responders (Figure 4a). IRF-1 -/- responders produced levels of IL-2 equal to those in IRF-1 sufficient MLC at 24 hours but levels increased between 48 and 72 hours to levels nearly three times of those observed in IRF-1 sufficient MLC ($p<0.001$). Results shown are

representative of three independent experiments. These experiments suggest that the regulation of CD4 T cell responses, and therefore IL-2 levels, by IFN-γ are mediated through IRF-1.

4.2 ANTI-PROLIFERATIVE EFFECTS OF IRF-1 IN MLC. If the increased IL-2 levels in MLC with IRF-1 -/- responders is associated with enhanced proliferation compared to IRF-1 sufficient MLC, then we should observe increased proliferation by MLC with IRF-1 -/- responders compared to IRF-1 sufficient MLC. MTT assays were performed as above to determine proliferation rates of the different MLCs. After 96 hours of culture proliferation in MLCs with IRF-1 -/- responders was increased approximately three-fold when compared to IRF-1 sufficient MLCs (Figure 4b) ($p<0.0005$). This assay is representative of three separate experiments. These results follow a similar pattern to those displayed by IFN-γ deficient MLCs (Figure 3n - ii) albeit to a lesser degree perhaps due to the decreased number of CD8 T cells.

4.3 Effects of IRF-1 on the Generation of Lytic Activity in MLC. Given that we had previously observed enhanced lytic activity every time IL-2 levels were increased, we were curious to determine whether this was also the case in MLC with IRF-1 -/- responders. To answer this question we performed ^{51}Cr release assays, as described above, and compared the lytic activity of wild-type effectors to those of IRF-1 -/- effectors after 5 days of culture. We found that despite the increased levels of IL-2 found in MLC with IRF-1 -/- responders, these cultures failed to generate any significant lytic activity (Figure 4c). This was a surprising result. We had not expected as dramatic of an

increase in lytic activity as in IFN-γ deficient MLC or MLC with IFN-γR1 -/- responders, given that IRF-1 -/- mice have decreased number of CD8 T cells, but we did expect these MLCs to generate some degree of lytic activity. These results are representative of three separate experiments.

IFN-γ production is also considered an effector function of T cells. To determine if this effector function was also abolished in IRF-1 -/- responders we assayed for IFN-γ levels in the supernatants every 24 hours by ELISA. We found that IFN-γ was produced by IRF-1 -/- responders, although levels were lower than those in IRF-1 sufficient MLC (Figure 4d) ($p<0.01$). This suggests that IRF-1 can enhance the amount of IFN-γ produced in a MLC, in agreement with previous studies (1).

Altogether these experiments argue that IRF-1 expression is necessary for the generation of CTL lytic activity and that this cannot be overcome by enhanced IL-2 production. Further, it shows that although IRF-1 expression is not required for IFN-γ production in MLC, it is required for optimal production of this cytokine.

4.4 THE ROLE OF IRF-1 IN T CELL ACTIVATION IN MLC. One reason for improper generation of CTL effectors in MLC with IRF-1 -/- responders despite increased IL-2 levels may be improper expression of CD25 on activated IRF-1 -/- CD8 T cells leading to an inability to receive IL-2. We determined expression of CD25 on T cells generated in IRF-1 sufficient MLC and MLC with IRF-1 -/- responders by flow cytometry after 5 days of culture. CD25 expression was enhanced on both wild-type and IRF-1 -/- T cells (Figure 4e). Levels of CD25 were higher on IRF-1 -/- CD4 and CD8 T cells compared to wild-type ($p<0.0001$), although decreased numbers of CD8 T cells

were still evident in MLC with IRF-1 -/- responders. This assay is representative of three separate experiments. These data rule out the possibility that improper CTL lytic activity is generated in MLC with IRF-1 -/- responders due to inappropriate expression of CD25. Further, these experiments strongly suggest that IRF-1 is critical for the generation of CTL effectors with lytic activity against allogeneic targets.

4.5 FIGURES

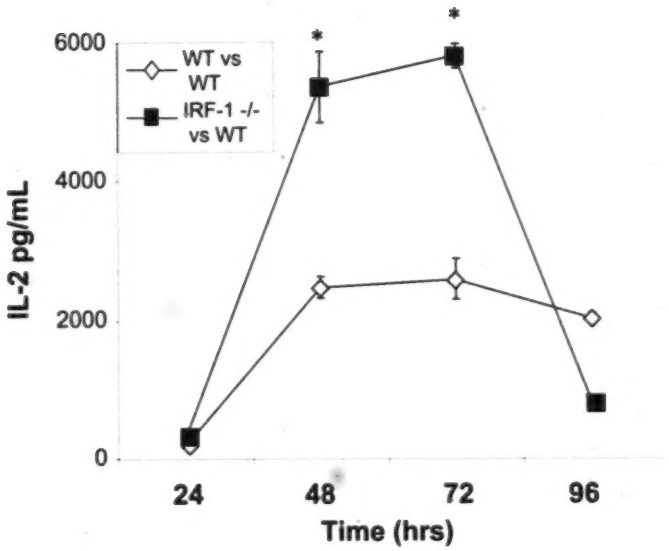

Figure 4 a. Effect of IRF-1 on IL-2 production in MLC. IL-2 levels in supernatants of IRF-1 sufficient MLC (WT vs WT) or MLC with IRF-1 -/- responders (IRF-1 -/- vs WT) were detected by ELISA. Results are expressed as mean IL-2 values ± SD. Each experiment included three separate MLC, composed of pooled stimulators from three mice and pooled responders from three mice. (* denotes significantly higher IL-2 levels at 48 and 72 hours, $p<0.001$ when compared to WT vs WT). Data shown is representative of one of three experiments.

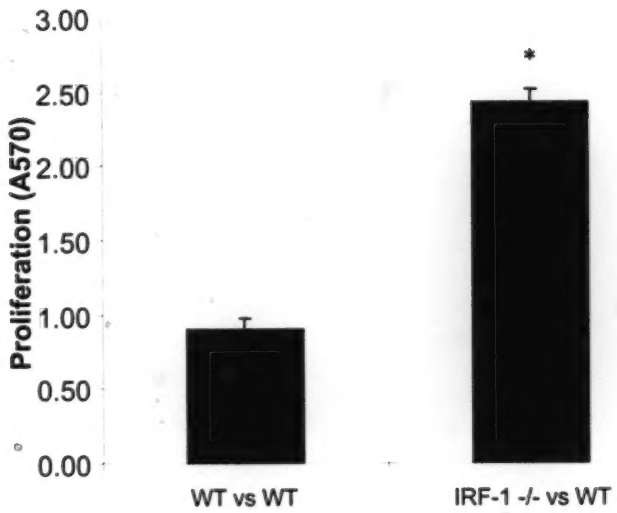

Figure 4 b. Antiproliferative effects of IRF-1 in MLC. Cells were seeded in 96-well plates in triplicate and proliferation measured after 96 hours of culture by the MTT assay. Proliferation is shown for IRF-1 sufficient MLC (WT vs WT) and MLC with IRF-1 -/- responders (IRF-1 -/- vs WT). Each experiment included 3 separate MLC composed of pooled stimulators from 3 mice and pooled responders from 3 mice. Results are expressed as mean values ± SD for one experiment. (* denotes significantly increased proliferation compared to WT vs WT, $p<0.0005$). Each MLC was performed in triplicate and represent three independent experiments.

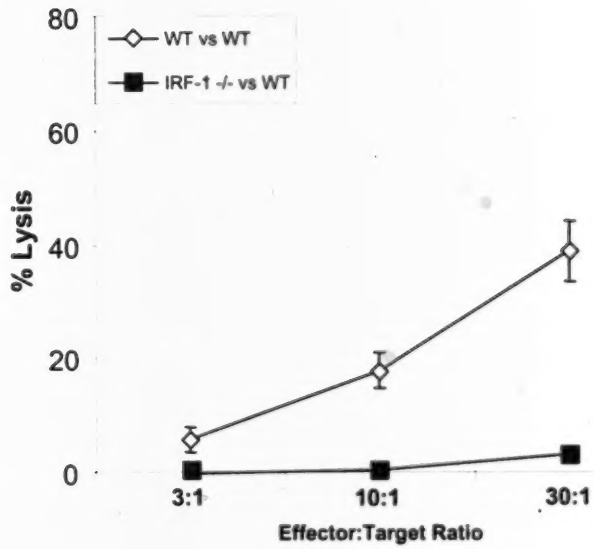

Figure 4 c. Effects of IRF-1 on the generation of lytic activity in MLC. Lytic activity was determined after five days in IRF-1 sufficient MLC (WT vs WT) or MLC with IRF-1 -/- responders (IRF-1 -/- vs WT). Each experiment included three separate MLC composed of pooled stimulators from three mice and pooled responders from three mice. Results show mean lysis ± SD of triplicate samples in one experiment and represent three independent experiments.

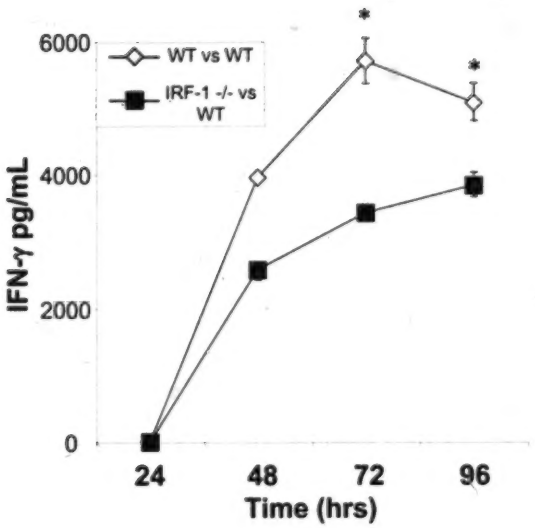

Figure 4 d. Effect of IRF-1 on IFN-γ production in MLC. IFN-γ levels in supernatants of IRF-1 sufficient MLC (WT vs WT) or MLC with IRF-1 -/- responders (IRF-1 -/- vs WT) were detected by ELISA. Each experiment included three separate MLC composed of pooled stimulators from three mice and pooled responders from three mice. Results are expressed as mean IFN-γ values ± SD for one experiment. (* denotes significantly higher IFN-γ levels at 72 and 96 hours, $p<0.01$ when compared to IRF-1 -/- vs WT). Assays performed in triplicate and represent three independent experiments.

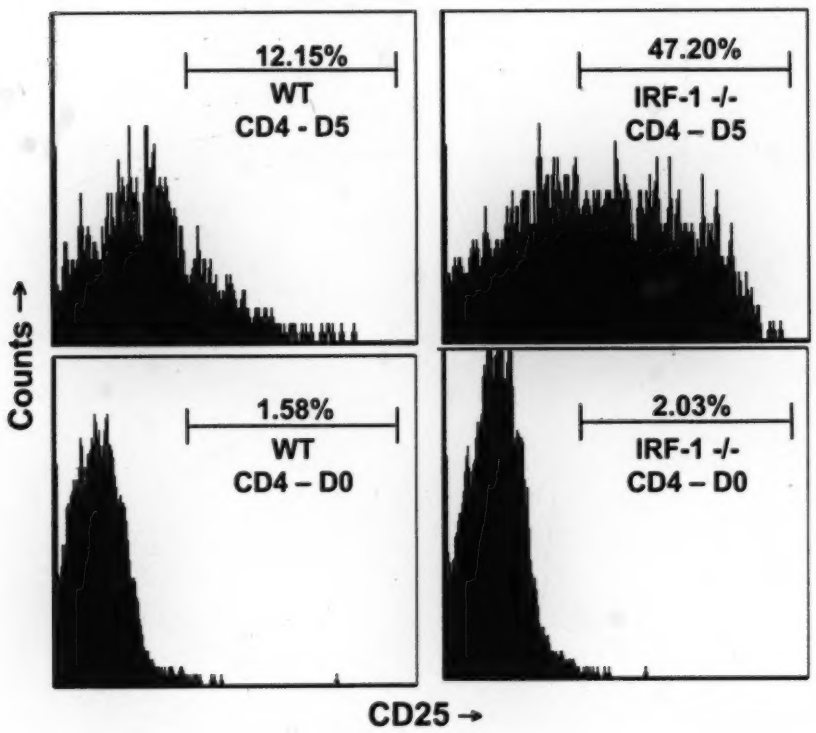

Figure 4 e. Effect of IRF-1 on CD25 expression in MLC. CD25 expression was determined by flow cytometry on CD4 T cells from IRF-1 sufficient (WT) or MLC with IRF-1 -/- responders before culture (D0) and after five days (D5). Each experiment included three separate MLC composed of pooled stimulators from three mice and pooled responders from three mice. Graphs show percent of CD4 T cells in one MLC. ($p<0.0001$ comparing % CD25+ CD4 T cells between WT and IRF-1 -/-). Data is representative of three individual mice per experiment and two independent experiments.

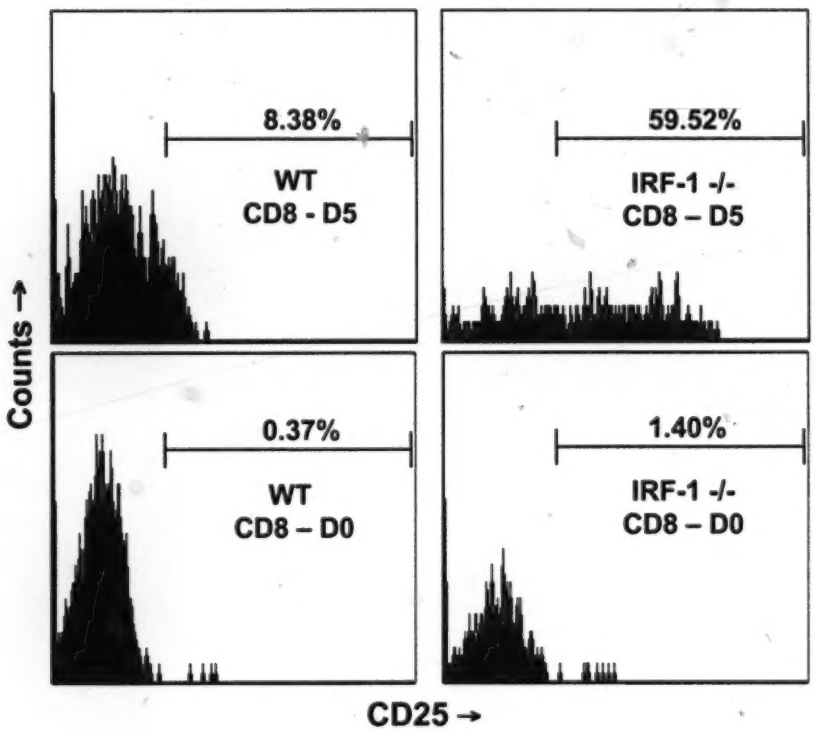

Figure 4 f. Effect of IRF-1 on CD25 expression in MLC. CD25 expression was determined by flow cytometry on CD8 T cells from IRF-1 sufficient (WT) or MLC with IRF-1 -/- responders before culture and after five days. Each experiment included 3 separate MLC composed of pooled stimulators from 3 mice and pooled responders from 3 mice. Graphs show percent of CD8 T cells. ($p<0.0001$ comparing % CD25+ CD8 T cells between WT and IRF-1 -/-). Data is representative of three individual mice per experiment and two independent experiments.

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CHAPTER 5

IN VIVO EFFECTS OF IFN-γ ON CTL GENERATION

The effects of IFN-γ in MLC may be different from those observed *in vivo*. Furthermore, since the effect observed in MLC centered on IL-2, and the role of IL-2 in *in vivo* CTL generation is debatable (1), we investigated whether our observations in MLC also applied *in vivo*.

5.1 EFFECTS OF IFN-γ IN CTL GENERATION IN *IN VIVO* ALLOIMMUNE RESPONSES. After having shown that IFN-γ is a negative regulator of CTL generation in MLC, we hypothesized that negative regulation of IL-2 production from CD4 T cells by IFN-γ was responsible for the increased *in vivo* lytic activity in IFN-γR1 -/- mice observed at the beginning of this project. This was particularly important given that the primary role for IL-2 in our proposed feedback loop and the ability of IL-2 -/- mice to develop *in vivo* CTL responses (2). As in our original studies we injected P815 cells expressing H-2^d into wild-type C57BL/6 or IFN-γR1 -/- mice expressing H-2^b. To induce alloimmune responses, we injected $5x10^6$ P815 cells subcutaneously and assessed CTL generation in the draining (axillary) lymph nodes and spleen after 7 days.

We estimated the strength of the CTL response generated in wild-type and IFN-γR1 -/- hosts using IFN-γ production upon *ex vivo* restimulation as an indication. Wild-type or IFN-γR1 -/- mice were injected with P815 cells suspended in sterile PBS or PBS alone subcutaneously in the interscapular region and draining lymph nodes and spleens

were collected after 7 days. Single cell preparations from each mouse were prepared for restimulation assays performed in 96-well plates. Lymph node cells and splenocytes from wild-type or IFN-γR1 -/- mice injected with PBS alone produced almost undetectable levels of IFN-γ (Figure 5a).

Proliferation or IFN-γ production following restimulation assays correlates with the number of effector T cells present in the population of interest, based on their ability to recognize a previously encountered antigen (3). In our case we used mitomycin C-treated P815 cells ($1x10^5$ cells/well), or CBA-derived T6SV cells ($H\text{-}2^k$) to determine allospecificity. Since the mice had been challenged with P815 cells, the number of CTL generated would be reflected in the amount of IFN-γ produced. Stimulator cells and effector cells were combined for 18 hours after which supernatants were collected and analyzed for IFN-γ levels by ELISA. Lymph node cells and splenocytes from wild-type or IFN-γR1 -/- mice injected with PBS alone produced almost undetectable levels of IFN-γ (Figure 5a). Restimulation of lymph node cells and splenocytes from mice injected with P815 cells showed that wild-type mice produced a significant response but this amounted to approximately one-fifth the strength of the responses observed in IFN-γR1 -/- mice, as measured by IFN-γ levels, suggestive of a greater population of alloreactive effector CD8 T cells. The production of IFN-γ during restimulation was allospecific since restimulation with T6SV tumor cells produced undetectable levels of IFN-γ. To confirm that IFN-γ was produced by CD8 T cells we performed IC staining for IFN-γ during restimulation assays. IC staining showed that there was no difference in the amount of IFN-γ-producing CD4 cells but a higher percent of IFN-γ producing CD8 cells in the spleens of IFN-γR1 -/- mice compared to wild-type (1.83% vs 0.86% of total CD3+

cells, respectively) (Figure 5b). These results are representative of three wild-type and three IFN-γR1 -/- mice in one experiment and two independent experiments.

As a second way to determine the number of CTL generated during alloimmune responses, we used expression of the activation marker CXCR3. During the differentiation to effectors, non-polarized T cells lose expression of homing chemokine receptors such as CCR7 and increase surface expression of the chemokine receptor CXCR3 which, in combination with other molecules, facilitates homing into inflamed tissues (4). We examined expression of CXCR3 on the cells used in the restimulation assays by flow cytometry (Figure 5c). The percent of CD8 T cells expressing CXCR3 in P815-challenged IFN-γR1 -/- mice was nearly twice of that found in wild-type mice in lymph nodes (14.6% of T cells vs 7.5%, respectively) and spleen (15.0% of T cells vs 8.9%, respectively). Results shown are representative of three individual mice and five independent experiments. Altogether these experiments suggest that a greater number of allospecific CTL are generated in IFN-γR1 -/- mice in response to allogeneic tumor challenge compared to wild-type mice.

5.2 RESPONSES TO VARYING AMOUNTS OF ALLOANTIGEN IN WILD-TYPE AND IFN-γR1 -/- HOSTS. IFN-γ affects CTL generation and T cell homeostasis, but it is also implicated in the recruitment of effector T cells to an inflamed site (5), as well as being a potent activator of innate immune cells (6). As a result of these effects, aside from effects on T cell generation, it is possible that the clearance of P815 cells would be affected in IFN-γR1 -/- mice due to either altered T cell recruitment or improper activation of innate immune cells. The direct effects of IFN-γ on tumor cells

are not a concern since IFN-γR1 -/- mice are capable of producing IFN-γ. Furthermore, although allogeneic tumor cell challenge leads to the generation of effector CTL, there is no direct evidence that these are the effector cells responsible for the destruction of the tumor (7).

To investigate whether the increases in CTL generation were not due to a difference in the amount of antigen present as a result of improper clearance from IFN-γR1 -/- mice, we performed P815 tumor challenges using varying number of cells. P815 cells were injected into wild-type and IFN-γR1 -/- mice as before but at three different doses: $5x10^6$ cells, $10x10^6$ cells, or $20x10^6$ cells per mouse. Each dose was performed as an individual experiment using three wild-type and three IFN-γR1 -/- mice. After seven days, draining lymph nodes and spleens were collected and restimulation assays performed as before (Figure 5d). The amount of IFN-γ produced in response to restimulation of wild-type lymph node cells increased in proportion to the number of P815 cells originally injected (linear regression, R=0.955, $p<0.01$). Lymph node cells from IFN-γR1 -/- mice on the other hand showed a strong response, even at the lowest number of P815 cells injected. The amount of IFN-γ produced by lymph node cells from IFN-γR1 -/- mice was higher than the amount produced by wild-type mice at each dose of P815 cells ($p<0.001$ in each experiment). However, the amount of IFN-γ produced by IFN-γR1 -/- lymph node cells was not affected by the increased numbers of P815 cells injected (linear regression, R=0.293, $p<0.05$).

Responses in the splenocytes followed a similar pattern to those from the lymph nodes, but the differences between wild-type and IFN-γR1 -/- mice were even more enhanced. The amount of IFN-γ produced by wild-type splenocytes increased

moderately with increasing number of P815 cells in a linear fashion (linear regression, R=0.999, $p<0.001$). IFN-γR1 -/- splenocytes again responded very strongly with the lowest number of P815 cells injected and responses again were not altered by an increasing number of P815 cells (linear regression, R=0.098, $p=0.18$). Thus, the response was much stronger in IFN-γR1 -/- mice at all doses of P815 cells compared to wild-type mice, particularly in the spleens of responding mice. These experiments demonstrate that the increased CTL generation observed in IFN-γR1 -/- mice is not affected by increased amounts of alloantigen at the initiation of the response and argue against increased antigen load as a reason for increased CTL generation.

5.3 THE ROLE OF IL-2 IN *IN VIVO* RESPONSES TO ALLOGENEIC TUMOR CHALLENGE. We had previously shown that the reason for the increased CTL generation in MLC with IFN-γR1 -/- responders was increased IL-2 levels. Thus we decided to examine IL-2 production *in vivo*. To determine if increased levels of IL-2 were produced in IFN-γR1 -/- mice, we performed restimulation assays using mitomycin C-treated splenocytes from DBA/2 mice as stimulators and spleen and lymph node cells from P815 challenged wild-type or IFN-γR1 -/- mice as responders. DBA/2 splenocytes were used, since we wanted to be sure to stimulate CD4 T cells via direct antigen presentation, and P815 cells lack expression of MHC class II molecules. Following 18 hours of restimulation, supernatant samples were taken and analyzed for IL-2 levels by ELISA (Figure 5e). The levels of IL-2 produced by lymph node cells from P815 challenged mice were higher than the levels produced by splenocytes for both wild-type and IFN-γR1 -/- mice. IL-2 was detectable with the restimulation of PBS-treated mice

but the levels were low (<200 pg/mL) and not different between wild-type and IFN-γR1 -/- mice. Lymph node cells from P815-treated IFN-γR1 -/- mice produced higher amounts of IL-2 compared to P815-treated wild-type mice (1756 pg/mL versus 613 pg/mL, respectively, $p<0.0001$). This difference was also observed with restimulation of wild-type and IFN-γR1 -/- splenocytes, although the increases over the amount of IL-2 produced by mice injected with PBS alone were less yet still significant ($p<0.0005$). Despite not directly assessing *in vivo* IL-2 production, these experiments provide evidence that increased amounts of IL-2 are produced in IFN-γR1 -/- mice compared to wild-type mice following allogeneic tumor challenge.

We had thus far shown that IFN-γ limits CTL generation *in vitro* as well as *in vivo* and evidence of increased IL-2 production *ex vivo*. Although IL-2 is not essential for CTL responses, it does exhibit a potent amplifying effect on CTL generation *in vivo* (2). If IL-2 is responsible for the increased generation of CTL in IFN-γR1 -/- mice, then neutralizing IL-2 during the immune response should generate results similar to those we observed when IL-2 was neutralized in MLC (Figure 3g). To neutralize IL-2 IFN-γR1 -/- mice were injected with rat anti-mouse IL-2 mAb (JES6-1A12, 150 μg per mouse) given i.p. 24 hours after P815 challenge. Isotype control (Rat IgG1, 150 μg per mouse) was given i.p. to control IFN-γR1 -/- mice 24 hours after challenge. Splenocytes from IFN-γR1 -/- mice given isotype control antibody produced higher amounts of IFN-γ in restimulation assays compared to wild-type splenocytes (20 ng/ml versus 6 ng/ml, respectively) (Figure 5f). IFN-γR1 -/- mice treated with anti-IL-2 showed a marked decrease in the amount of IFN-γ produced upon restimulation compared to isotype control mice (11 ng/ml versus 20 ng/ml, respectively. $p<0.0001$). IL-2 neutralization also

decreased the percent of CXCR3+ CTL compared to that observed on isotype treated animals and to similar levels as wild-type mice (Figure 5g). These data indicate that the increased generation of CTL effectors in IFN-γR1 -/- mice in response to allostimulation requires IL-2, similar to our *in vitro* findings.

5.4 EFFECT OF THE CHANGE OF ALLOGENEIC TUMOR CHALLENGE SITE. The immune response generated can at times be affected by the site where antigen is encountered. Also, since the mice used in these studies are on a complete C57BL/6 background instead of the 129/B6 hybrid background we originally used, it was important to confirm the same phenotype observed in 129/B6 IFN-γR1 -/- mice (Figure 3a). We determined the percent of CXCR3+ CTL and amount of lytic activity generated in wild-type C57BL/6 and IFN-γR1 -/- C57BL/6 mice 7 days following peritoneal injection of $5x10^6$ P815 cells. Similar to our initial findings, splenocytes from IFN-γR1 -/- C57BL/6 mice displayed greater lytic activity compared to wild-type C57BL/6 mice as determined by ^{51}Cr-release assay of P815 target cells at all effector:target ratios tested (Figure 5h). Coinciding with the enhanced lytic activity, we also observed an increase in the percent of CD8 T cells expressing the activation marker CXCR3 in IFN-γR1 -/- C57BL/6 splenocytes (GRKO Spleen) compared to wild-type C57BL/6 splenocytes (WT Spleen) (23.46% versus 4.6% of T cells, respectively, $p<0.005$) (Figure 5i). Data shown is representative of two independent experiments, each with three mice per group.

Together these results demonstrate three important points: 1) CTL responses to allogeneic P815 tumors are increased in IFN-γR1 -/- C57BL/6 mice compared to wild-type C57BL/6 mice regardless of the site of injection, 2) the percent of CXCR3+ CD8 T

can be indicative of the amount of lytic activity generated *in vivo*, and 3) CTL responses in IFN-γR1 -/- mice on a pure C57BL/6 background are similar to those elicited in IFN-γR1 -/- mice on a 129/B6 background in response to allogeneic tumor challenge.

5.5 FIGURES

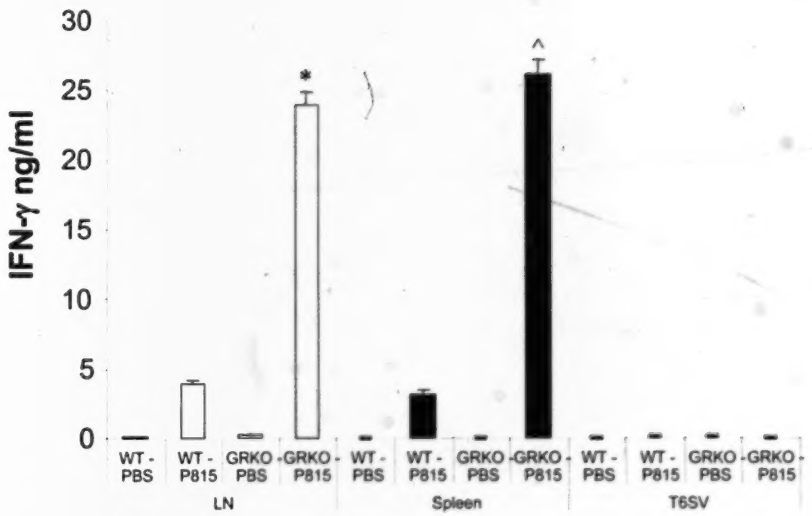


Figure 5 a. Assessment of allospecific IFN-γ production upon restimulation following allogeneic challenge. Wild-type (WT) or IFN-γR1 -/- (GRKO) mice were injected with P815 cells subcutaneously or PBS and cells were harvested after seven days. Cells from draining lymph node and spleen were stimulated with mitomycin-treated P815 stimulator cells for 18 hours. Supernatants were analyzed for IFN-γ content by ELISA. T6SV stimulation of spleen cells was used as control. Results show mean IFN-γ values ± SD of three mice per treatment and represent five independent experiments. (* denotes significantly increased IFN-γ levels compared to LN WT-P815, $p<0.0001$, ^ denotes significantly increased IFN-γ levels compared to spleen WT-P815, $p<0.0001$). ELISA assays were performed in triplicate.

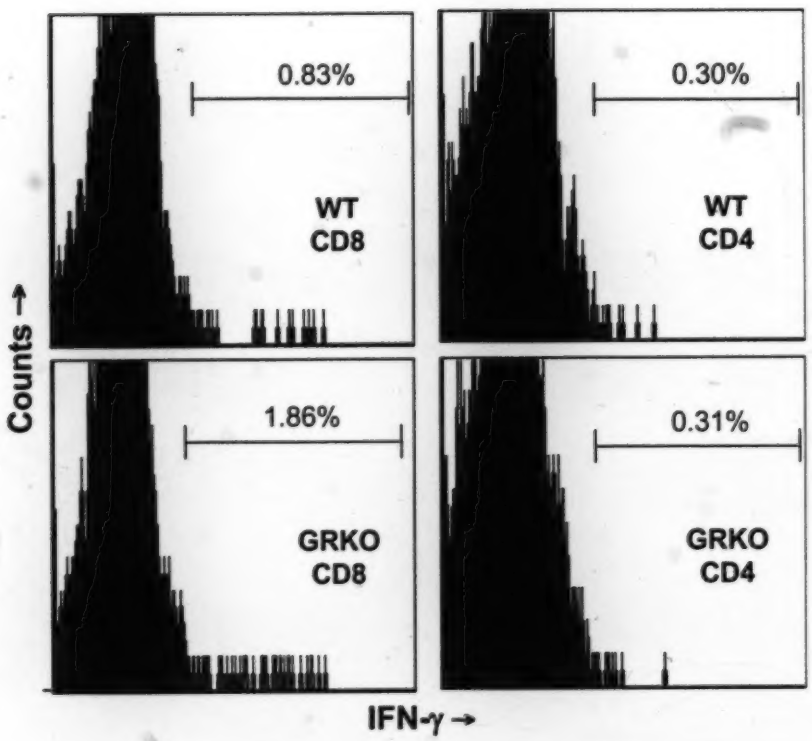


Figure 5 b. Allogeneic responses in IFN-γR1 -/- mice lead to increased numbers of IFN-γ producing cells. IFN-γR1 -/- (GRKO) or wild-type (WT) mice injected with P815 cells subcutaneously and spleens collected after seven days. Intracellular levels of IFN-γ in spleen cells restimulated with mitomycin-treated P815 cells were determined by flow cytometry. Graphs show percent of CD3+ cells. Data is representative of three mice in one experiment and two independent experiments.

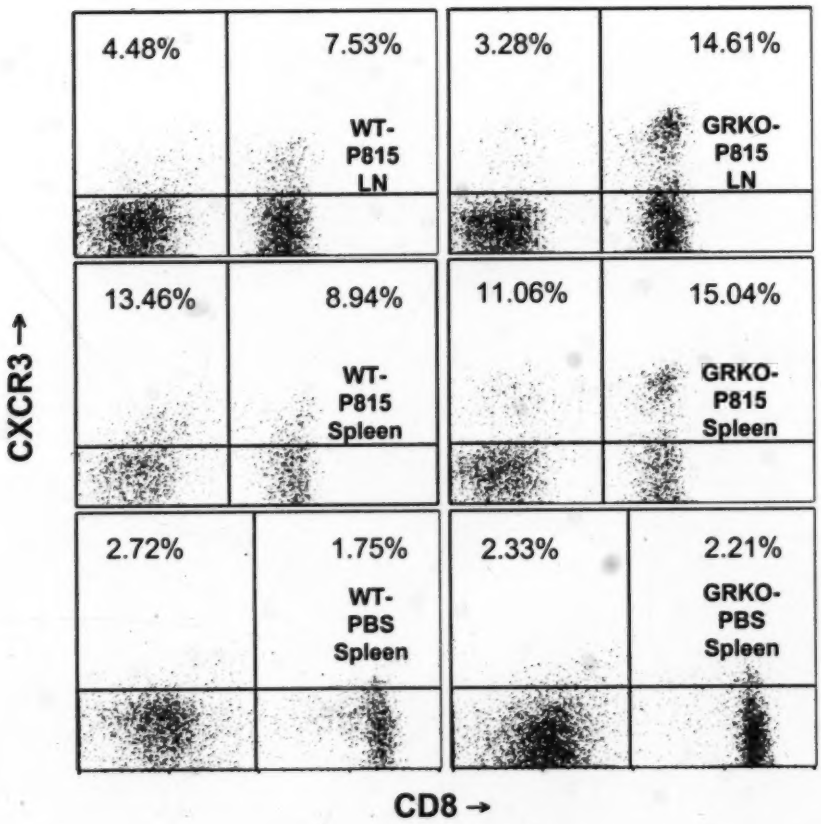
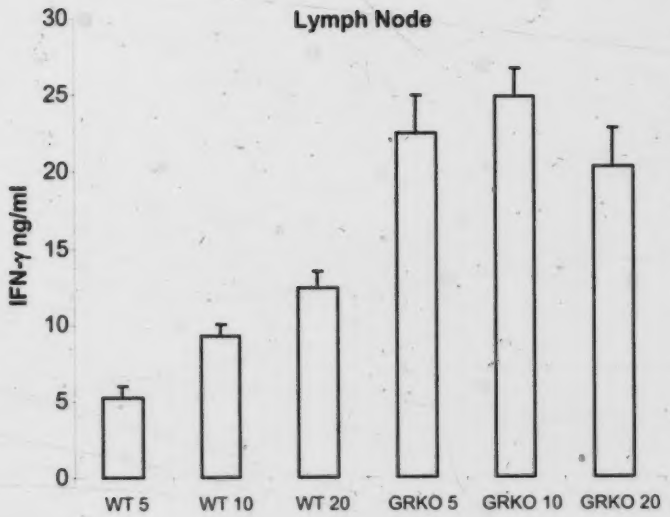


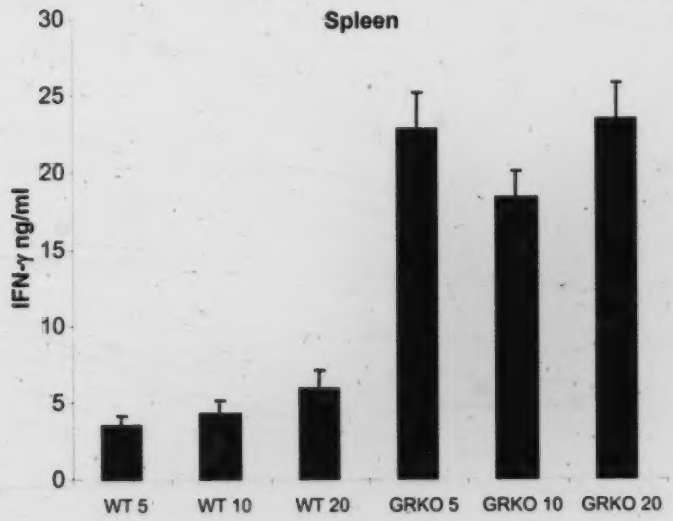
Figure 5 c. CXCR3 expression on CD8 cells following allogeneic challenge. IFN-γR1 -/- (GRKO) or wild-type (WT) mice were injected with P815 cells or sterile PBS subcutaneously and draining lymph nodes and spleens collected after seven days. Expression of CXCR3 on CD3+8+ cells was determined by flow cytometry. Graphs show percent of CD3+ cells. ($p<0.01$ in LN, and $p<0.005$ in spleen comparing %CD8+CXCR3+ T cells between wild-type and IFN-γR1 -/- mice). Data is representative of three individual mice per experiment and five independent experiments.

Figure 5 d. Assessment of allospecific CTL generated in response to varying amounts of alloantigen. Wild-type (WT) or IFN-γR1 -/- (GRKO) mice were injected with 5, 10, or 20 X 10^6 P815 cells subcutaneously and cells were harvested after seven days. Cells from draining lymph node i) and spleen ii) were stimulated with mitomycin-treated P815 stimulator cells for 18 hours. Supernatants were analyzed for IFN-γ content by ELISA. Results show mean IFN-γ values ± SD of samples in triplicate. Each dose of P815 cells was performed in separate experiments using three mice per group. ($p<0.001$ for Lymph Node and $p<0.005$ for Spleen comparing wild-type to IFN-γR1 -/- in each experiment). Data is representative of at least two independent experiments.

i)


Lymph Node
IFN-γ ng/ml
30
25
20
15
10
5
0
WT 5
WT 10
WT 20
GRKO 5
GRKO 10
GRKO 20

ii)


Spleen
IFN-γ ng/ml
30
25
20
15
10
5
0
WT 5
WT 10
WT 20
GRKO 5
GRKO 10
GRKO 20

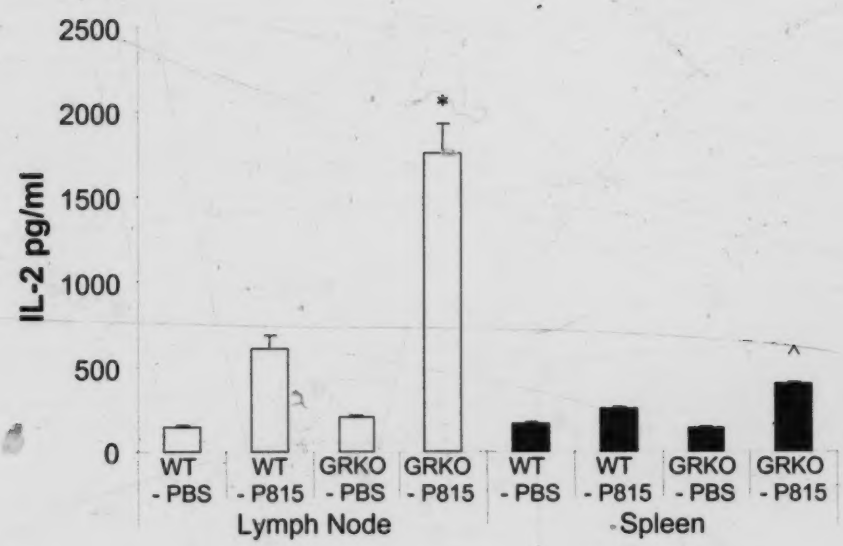


Figure 5 e. Assessment of *ex vivo* IL-2 production following allogeneic challenge. Wild-type (WT) or IFN-γR1 -/- (GRKO) mice were injected with P815 cells or PBS subcutaneously and cells were harvested after seven days. Cells from draining lymph node and spleen were stimulated with mitomycin-treated DBA/2 spleen cells for 18 hours. Supernatants were analyzed for IL-2 content by ELISA. Results show mean IL-2 values ± SD of three mice per treatment and represent five independent experiments. (* denotes significantly increased IL-2 levels compared to LN WT-P815, $p<0.0001$, ^ denotes significantly increased IL-2 levels compared to spleen WT-P815, $p<0.0005$). ELISA assays were performed in triplicate. Data represents three independent experiments.

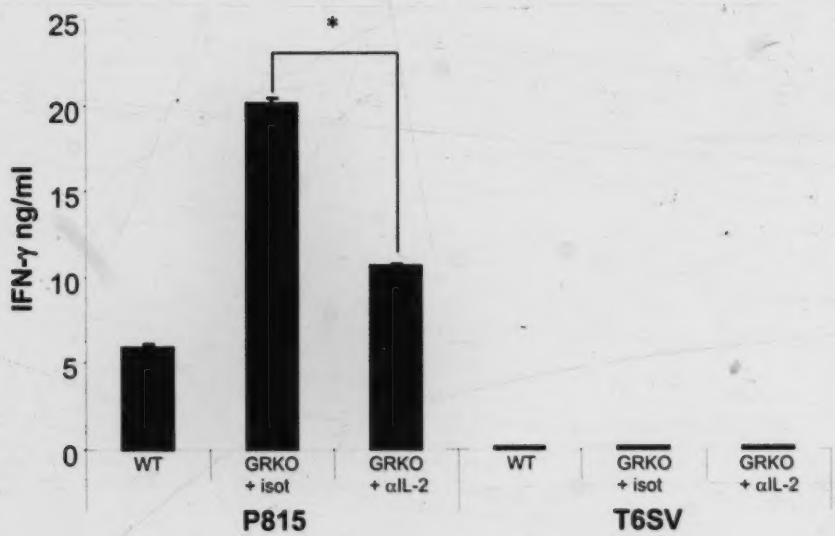


Figure 5 f. Effect of *in vivo* IL-2 neutralization on allospecific IFN-γ production upon restimulation following allogeneic challenge. Wild-type (WT) or IFN-γR1 -/- mice were injected with P815 cells subcutaneously and IFN-γR1 -/- mice were treated with 250 μg of anti-IL-2 (GRKO + αIL-2) or isotype control antibody (GRKO + isot). Spleens were harvested after seven days. Spleen cells were stimulated with mitomycin-treated P815 stimulator cells for 18 hours. Supernatants were analyzed for IFN-γ content by ELISA. T6SV stimulation served as control. Results show mean IFN-γ values ± SD of three mice per treatment and represent three independent experiments. (* denotes significantly decreased IFN-γ levels, $p<0.0001$). ELISA assays were performed in triplicate.

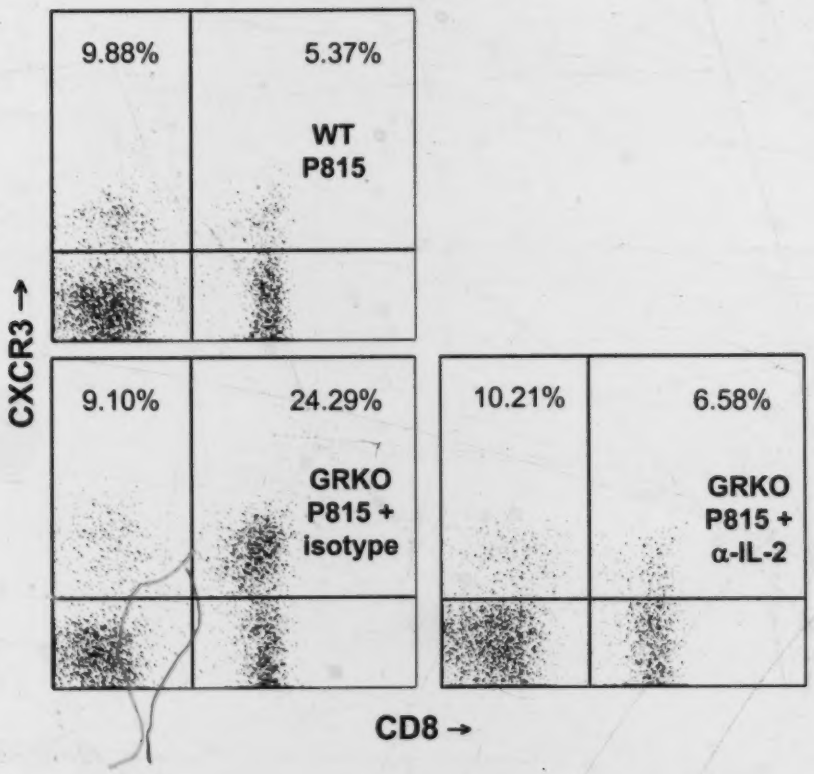


Figure 5 g. Effect of *in vivo* IL-2 neutralization on CTL generation following allogeneic challenge. Wild-type (WT) or IFN-γR1 -/- mice were injected with P815 cells subcutaneously and IFN-γR1 -/- mice were treated with anti-IL-2 (GRKO P815 + αIL-2) or isotype control antibody (GRKO P815 + isotype). Spleens were harvested after seven days. Expression of CXCR3 on CD3+8+ cells was determined by flow cytometry. Graphs show percent of total T cells. (p<0.001 comparing %CD8+CXCR3+ T cells between GRKO + isot and GRKO + αIL-2). Data is representative of three individual mice per experiment and three independent experiments.

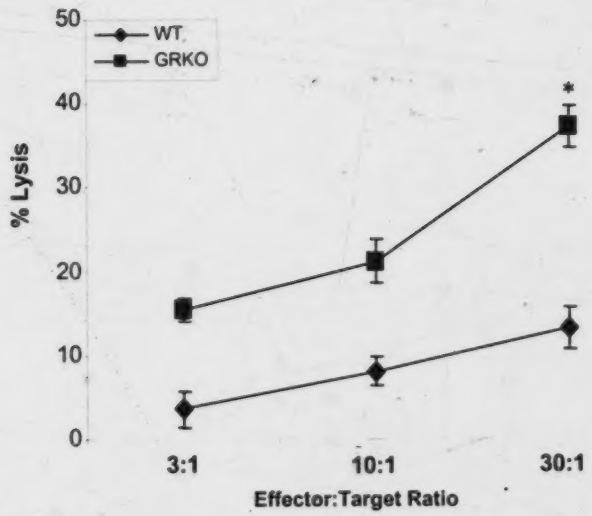


Figure 5 h. Effect of IFN-γ on *in vivo* generation of lytic activity. Mice were injected with P815 cells intraperitoneally and spleens collected after seven days. Lytic activity was determined after five days using IFN-γR1 -/- (GRKO) or wild-type (WT) spleen cells as effectors. Results show mean lysis ± SD of triplicate samples and represent two independent experiments. (* denotes significantly increased lytic activity compared to WT at 30:1 Effector:Target Ratio, $p<0.0001$). Data shown is representative of two experiments.

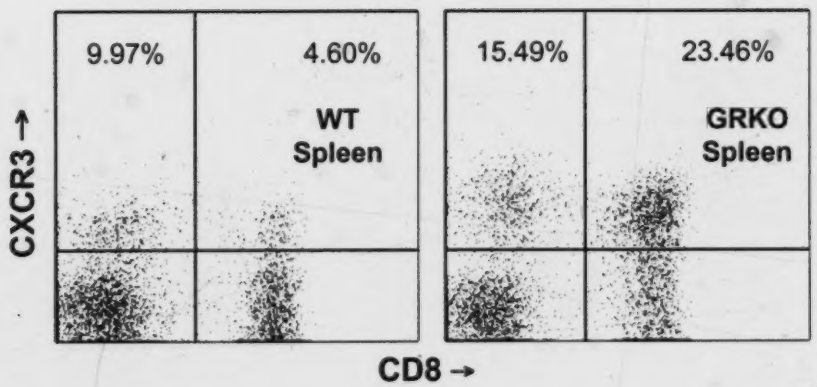


Figure 5 i. CXCR3 expression on CD8 cells following allogeneic challenge. IFN-γR1 -/- (GRKO) or wild-type (WT) mice were injected with P815 cells intraperitoneally and spleens collected after seven days. Expression of CXCR3 on CD3+8+ cells was determined by flow cytometry. Graphs show percent of CD3+ cells. ($p<0.005$ comparing %CD8+CXCR3+ T cells between WT and GRKO). Data is representative of three individual mice per experiment and two independent experiments.

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CHAPTER 6

CHANGES IN THE INFLAMMATORY COMPARTMENT INDUCED BY IFN-γ

IFN-γ affects chemokine and adhesion molecule expression on various cells of an inflamed site and thus can help define the cellular infiltrate. Previous results in this lab and those of others demonstrate that IFN-γ induces expression of the chemokines CXCL9-11, and RANTES and suppresses chemokines that recruit granulocytes and monocytes (1-3). Thus the presence of IFN-γ seems to promote activated T cell recruitment, while at the same time limiting the recruitment of monocytes and granulocytes normally associated with allergic and other inflammatory responses.

6.1 DETECTION OF IFN-γ AND EFFECTS OF CXCL9 EXPRESSION. We sought to analyze the cellular make-up of the infiltrate recruited in wild-type, IFN-γ -/- and IFN-γR1 -/- mice rejecting allogeneic tumors in their peritoneum. Mice were injected with $5x10^6$ P815 cells i.p. and cells in the peritoneal lavage were analyzed by flow cytometry after seven days. Supernatants from peritoneal lavages were used in ELISA assays to determine IFN-γ and CXCL9 levels. IFN-γ was easily detectable in peritoneal supernatants of wild-type and IFN-γR1 -/- mice (10.6 ± 1.2 ng/ml SD and 7.7 ± 1.6 ng/ml SD, respectively) (Figure 6a). Peritoneal supernatants of IFN-γ -/- mice showed no detectable levels of IFN-γ as expected. CXCL9 levels were detectable only in

wild-type supernatants (2.4 ng/mL ± 0.2, SD of CXCL9) demonstrating that IFN-γ and IFN-γR1 are absolutely necessary for expression of this chemokine (Figure 6b). Data shown is representative of two independent experiments each with three animals per group.

6.2 RECRUITMENT OF CXCR3+ T CELLS IN IFN-γR1 -/- MICE. Having shown that a higher percent of CXCR3+ CTL are generated in IFN-γR1 -/- mice, we sought to determine whether these cells would be recruited to the challenge site given that expression of peritoneal CXCL9, and presumably CXCL10-11 also, was severely reduced compared to wild-type mice. We determined by flow cytometry the percent of T cells expressing CXCR3 and CD8 in the peritoneum of wild-type and IFN-γR1 -/- mice seven days after tumor cell challenge. We found that CXCR3+ CTL were recruited into the peritoneum of challenged mice despite the lack of IFN-γ effects (Figure 6c-i). CXCR3+ T cells, CD8 and presumably CD4, were recruited into the peritoneum of wild-type and IFN-γR1 -/- mice. Surprisingly, a higher percent of T cells (both CD8+ and CD8-) were CXCR3+ in IFN-γR1 -/- mice compared to wild-type mice (68.7 % versus 45.2% of T cells, respectively. $p<0.0001$) (Figure 6c-i). However, when the total number of CD8 T cells in the peritoneum was calculated we found no significant difference between wild-type and IFN-γR1 -/- mice, although there was a trend for increased CD8 T cells in IFN-γR1 -/- mice (Figure 6c-ii). This data demonstrates that although CXCR3+ CTL can be recruited to an inflammatory site in the absence of IFN-γ effects, the total numbers of CXCR3+ CTL may not necessarily reflect the numbers generated in SLOs.

6.3 IFN-γ ALTERS THE CELLULAR INFILTRATE IN RESPONSE TO ALLOGENEIC TUMOR CELLS. The cell types recruited to an inflamed site are altered by the presence or absence of IFN-γ. Although this is already established in a number of models of pathogenic infections we tested if cell type recruitment is altered in alloimmune responses in IFN-γR1 -/- mice. Based on other studies in the lab, we postulated that the number of macrophages and granulocytes, including eosinophils, would be increased in the absence of IFN-γ effects. To test this, we analyzed the number of peritoneal cells expressing CD11b (Mac-1) by flow cytometry. The data is shown as the percent of peritoneal cells outside of the lymphocyte and granulocyte gates, as determined by the forward and side scatter profile, that express CD11b (Figure 6d). A higher percent of CD11b+ cells were found in the peritoneum of IFN-γR1 -/- mice compared to wild-type mice (44.71% versus 18.33%, respectively, $p<0.0001$). CD11b is primarily a monocyte/macrophage marker but it is also expressed on some granulocytes. We looked at the percent of CD11b+ cells that also express the macrophage specific marker F4/80 (4) (Figure 6e). The majority of CD11b+ cells also expressed F4/80 and did not differ between wild-type and IFN-γR1 -/- mice (81.07% and 79.5%, respectively). All F4/80+ cells were also CD11b+, confirming the specific expression of F4/80 on macrophages and the more promiscuous expression of CD11b (5). This data confirms that the majority of the increased numbers of CD11b+ cells in IFN-γR1 -/- mice are macrophages.

To examine for the presence of granulocytes we examined the side scatter profile of Ly-6G^{hi} cells from the peritoneal lavage of wild-type and IFN-γR1 -/- mice by flow cytometry. Ly-6G is a common granulocyte marker (also found on macrophages at low

levels) (6). We considered cells that expressed high levels of Ly-6G and had a high side scatter to be granulocytes. A much larger population of granulocytes was present in the lavage of IFN-γR1 -/- mice compared to wild-type mice ($10.7x10^7$ versus $0.3x10^7$, respectively. $p<0.05$), although a large amount of variability was observed in IFN-γR1 -/- mice (Figure 6f). Data represents two independent experiments each with three animals per group.

6.4 THE EFFECT OF IFN-γ ON THE ABILITY TO CLEAR ALLOGENEIC TUMOR CELLS. To assess the ability of IFN-γR1 -/- and wild-type mice to eliminate the allogeneic tumor, we determined the number of P815 cells remaining after seven days. P815 cells were identified in the peritoneal lavage as cells that express H-2^d and lack expression of the macrophage marker F4/80. Exclusion of F4/80+ cells was necessary, since we had observed that some macrophages acquired H-2^d, presumably through direct contact with P815 cells. Calculation of the total number of P815 cells remaining showed that many more P815 cells remained in the peritoneum of IFN-γR1 -/- mice compared to wild-type mice ($27.8x10^6$ versus $4.1x10^6$, respectively. $p<0.05$) (Figure 6g). Once again, like in the number of granulocytes, there was great variability in the number of P815 cells remaining in IFN-γR1 -/- mice. Data shown is representative of two independent experiments each with 3 animals per group.

6.5 FIGURES

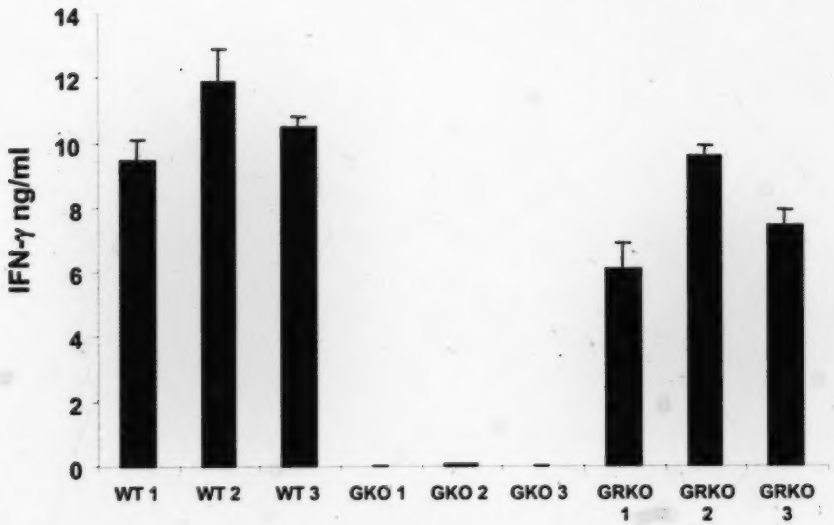


Figure 6 a. Presence of IFN-γ in the peritoneal cavity following allogeneic challenge. IFN-γR1 -/- (GRKO), IFN-γ -/- (GKO), and wild-type (WT) mice were injected with P815 cells i.p. and peritoneal lavage collected after seven days. Lavage fluid was analyzed for IFN-γ content by ELISA. Results show mean IFN-γ values ± SD of triplicate samples for individual mice and represent two independent experiments.

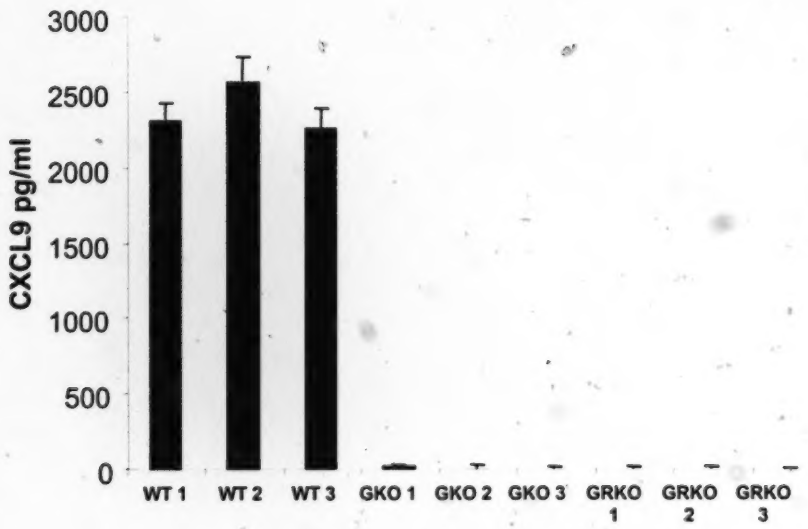
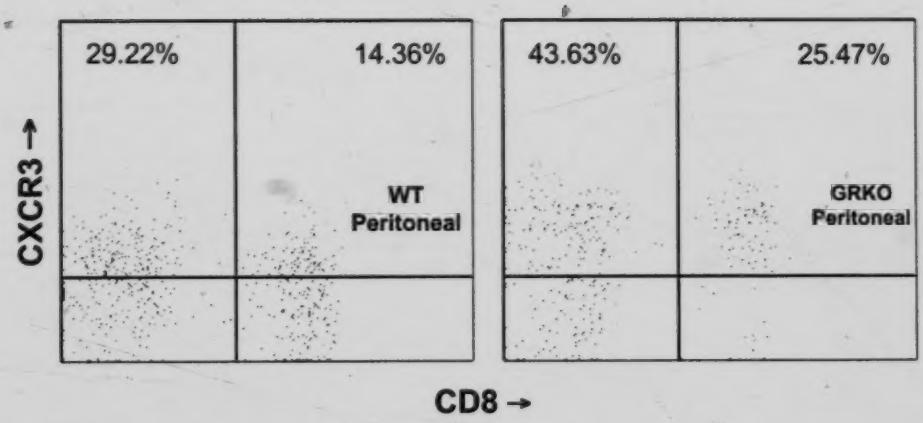


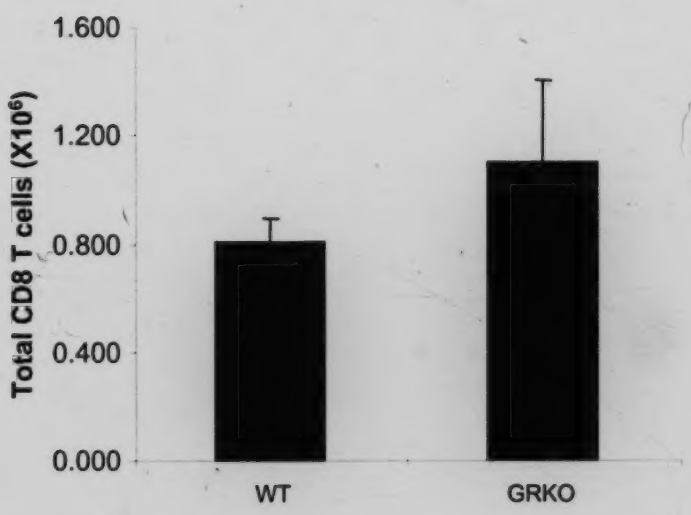
Figure 6 b. Presence of CXCL9 in the peritoneal cavity following allogeneic challenge. IFN-γR1 -/- (GRKO), IFN-γ -/- (GKO), and wild-type (WT) mice were injected with P815 cells i.p. and peritoneal lavage collected after seven days. Lavage fluid was analyzed for CXCL9 content by ELISA. Results show mean CXCL9 values ± SD of triplicate samples for individual mice and represent two independent experiments.

Figure 6 c. Presence of CXCR3+ T cells in the peritoneal cavity following allogeneic challenge. IFN-γR1 -/- (GRKO) and wild-type (WT) mice were injected with P815 cells intraperitoneally and peritoneal lavage collected after seven days. i) Expression of CXCR3 on CD3+8+ cells in the peritoneal lavage was determined by flow cytometry. Graphs show percent of total T cells. (p<0.0001 comparing % CXCR3+ T cells between WT and GRKO). Data is representative of three individual mice per experiment and two independent experiments. ii) Calculation of total CD8 T cell numbers in the peritoneal lavage. (p=0.18 comparing % CXCR3+ T cells between WT and GRKO). Data is representative of three individual mice in two independent experiments.

A)


29.22%
14.36%
43.63%
25.47%
WT
Peritoneal
GRKO
Peritoneal
CXCR3 →
CD8 →

B)


1.600
1.200
0.800
0.400
0.000
Total CD8 T cells (X10⁶)
WT
GRKO

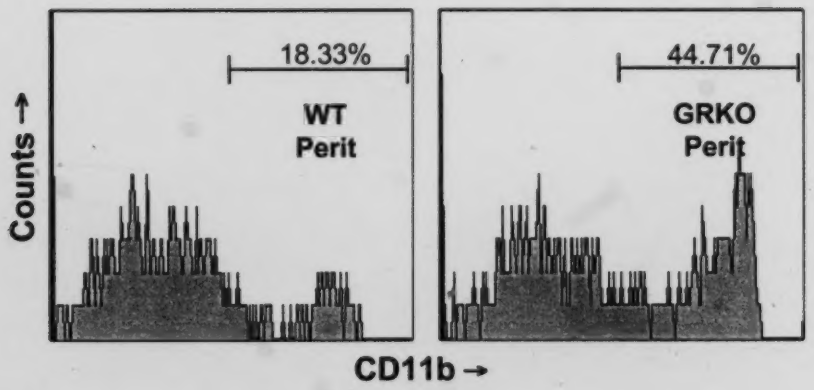


Figure 6 d. Effects of IFN-γ on peritoneal macrophages following allogeneic challenge. IFN-γR1 -/- (GRKO) and wild-type (WT) mice were injected with P815 cells intraperitoneally and peritoneal lavage collected after seven days. Number of CD11b macrophages in the peritoneal lavage was determined by flow cytometry. Graphs show percent of peritoneal cells outside of the lymphocyte and granulocyte gates. ($p<0.0001$ comparing % CD11b+ cells between WT and GRKO). Data is representative of three individual mice per experiment and two independent experiments.

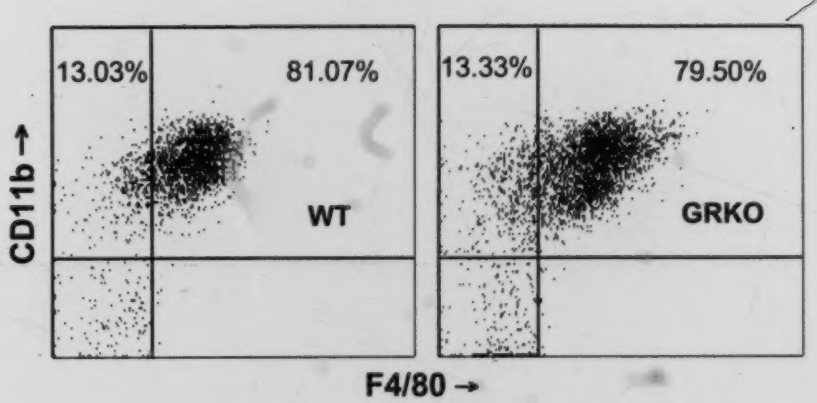


Figure 6 e. Effects of IFN-γ on macrophage marker expression in peritoneal microphages following allogeneic challenge. IFN-γR1 -/- (GRKO) and wild-type (WT) mice were injected with P815 cells intraperitoneally and peritoneal lavage collected after seven days. Macrophages were purified by removing CD3+, CD19+, H-2d+, Ly6G+ cells by FACS. Number of CD11b+F4/80+ cells remaining was determined by flow cytometry. Graphs show percent of cells outside of the lymphocyte and granulocyte gates. Data is representative of two independent experiments.

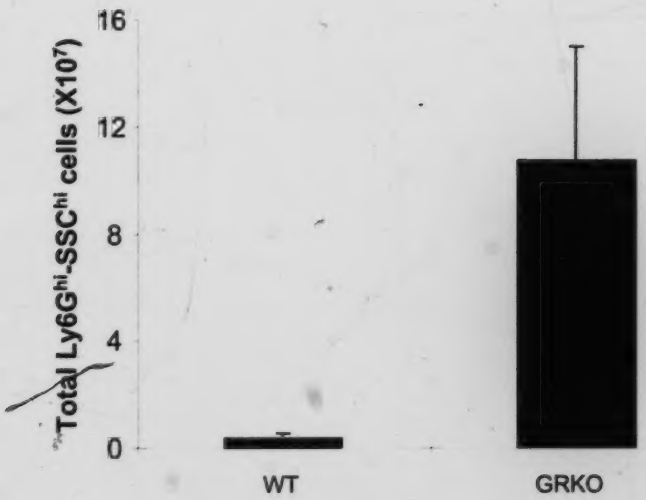


Figure 6 f. Effects of IFN-γ on the number of granulocytes in the peritoneum following allogeneic challenge. IFN-γR1 -/- (GRKO) and wild-type (WT) mice were injected with P815 cells intraperitoneally and peritoneal lavage collected after seven days. Number of Ly6G+ cells in the peritoneal lavage outside of the lymphocyte and monocyte/macrophage gates was determined by flow cytometry. Graph shows total number of $Ly6G^{hi}$ cells with a high side scatter (SSC^{hi}). ($p<0.05$ comparing total number of $Ly6G^{hi}$-SSC^{hi} cells between WT and GRKO). Data is representative of three individual mice per experiment and two independent experiments.

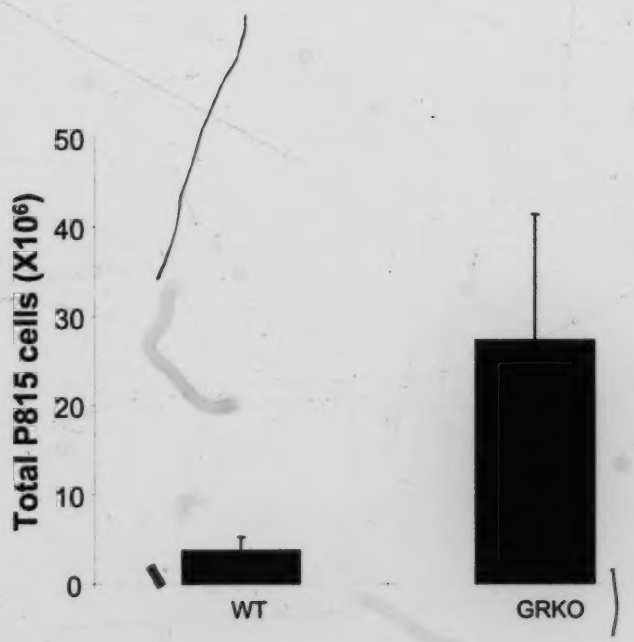


Figure 6 g. Effects of IFN-γ on the clearance of allogeneic tumor cells in the peritoneum. IFN-γR1 -/- (GRKO) and wild-type (WT) mice were injected with P815 cells intraperitoneally and peritoneal lavage collected after seven days. Number of H-2^d+F4/80- cells in the peritoneal lavage was determined by flow cytometry. Graphs show the calculated total number of H-2^d+F4/80- P815 cells in the peritoneum. ($p<0.05$ comparing total number of H-2d+F4/80- cells between WT and GRKO). Data is representative of three individual mice per experiment and two independent experiments.

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CHAPTER 7

GENERAL DISCUSSION

7.1 SUMMARY

The finding that allografts are rapidly rejected in IFN-γ -/- hosts was unexpected and allowed us to propose the hypothesis that IFN-γ limits CTL generation during alloimmune responses. Initial characterization of IFN-γ -/- mice demonstrated increases in CTL lytic activity in MLC supporting our hypothesis. Our preliminary *in vivo* experiments demonstrated a marked increase in lytic activity in IFN-γR1 -/- mice, suggesting that IFN-γ is involved in the generation of lytic activity both *in vitro* and *in vivo*. It was the intention of this project to elucidate the mechanism by which IFN-γ could limit CTL generation during alloimmune responses.

We first established two related but separate MLCs by which we could study the effects of IFN-γ on CTL generation. Using IFN-γR1 -/- responders, we were able to show increased lytic activity in these MLC as well as in IFN-γ deficient MLC. Both of these showed enhanced lytic activity generation compared to other studies which used wild-type stimulators along with IFN-γ -/- responders. When effectors generated in these MLC were analyzed, we found an increased percent of activated CD8 T cells when IFN-γ effects were absent. We then compared IL-2 levels in the supernatants of the MLCs and found they were also increased in all cases where IFN-γ effects were altered. Neutralization of IL-2 in IFN-γ deficient MLC reduced lytic activity to wild-type levels. Further, the addition of IL-2 to wild-type MLC increased their lytic activity to match

those of effectors generated in the absence of IFN-γ. Overall percent of CTL generated in MLCs was directly proportional to the amount of IL-2 present.

The regulation of IL-2 levels by IFN-γ was examined. Increases in IL-2 appeared to be the basis for the enhanced lytic activity observed in the absence of IFN-γ. Using purified CD4 or CD8 cells from wild-type or IFN-γR1 -/- mice as responders we identified CD4 cells as being the principal cells affected by IFN-γ leading to increased IL-2 production and lytic activity. This was not a direct effect of IFN-γ on IL-2 transcription, but more simply a transient effect on CD4 T cell proliferation since IL-2 levels were equal after 24 hours of stimulation. The anti-proliferative effect of IFN-γ was mediated through IRF-1, since IRF-1 -/- responders also produced higher levels of IL-2. However, no lytic activity was generated in these cultures, suggesting that CD8 T cells require IRF-1 to become cytolytic effectors. Experiments examining the differing roles of IRF-1 on CD4 versus CD8 T cell function are still needed.

We studied *in vivo* effects of IFN-γ or IFN-γR1 deficiency using allogeneic tumor cells as antigen. We demonstrated an increase in CTL effectors generated in spleens and draining lymph nodes of IFN-γR1 -/- mice compared to wild-type. We showed that restimulation of lymph node cells with antigen led to increased production of IL-2 by IFN-γR1 -/- cells. *In vivo* IL-2 neutralization in IFN-γR1 -/- mice decreased CTL effector generation similar to levels observed in wild-type mice. Surprisingly, the increased generation of CTL in SLOs did not translate into increased recruitment into the inflamed site. Moreover, this was not due to the lack of recruitment of CXCR3+ CTL, despite decreased expression of IFN-γ-inducible chemokines. Altogether these data support a

model by which IFN-γ produced by effector T cells feeds back on CD4 T cells to limit their proliferation resulting in decreased IL-2 levels and CTL generated.

Moreover, the ultimate elimination of antigen-bearing tumor cells was enhanced by IFN-γ. Although the number of CTL in the inflammatory site of IFN-γR1 -/- mice was equal to that of wild-type mice, the number of P815 cells remaining in wild-type mice was markedly lower than in IFN-γR1 -/- mice. We also noted that IFN-γ alters the cellular infiltrate at the affected site. Increased numbers of macrophages and granulocytes were evident in peritoneal lavages from IFN-γR1 -/- mice compared to wild-type mice. Whether this is a result of the altered chemokine milieu or differential adhesion molecule expression in the absence of IFN-γ effects will require further investigation.

7.2 IL-2 PRODUCTION AND LYTIC ACTIVITY IN THE ABSENCE OF IFN-γ OR IFN-γR1

Given the many non-redundant effects of IFN-γ in the immune response, immunologists predicted that its absence would produce an immunodeficient phenotype. The generation of IFN-γ -/- mice permitted the examination of the role of IFN-γ in the immune response, and revealed that, although it was important in macrophage activation and NK cell cytolysis, many T cell functions were enhanced rather than reduced (1). Lytic activity was modestly increased in MLC with IFN-γ -/- responders with wild-type stimulators compared to wild-type MLC. Our results using allogeneic tumor challenge in

displayed more drastic differences between wild-type and IFN-γR1 -/- mice in the amount of lytic activity generated, suggesting that perhaps the full phenotype of IFN-γ had not been completely elucidated.

Although the ability of T cells to produce maximal amounts of IFN-γ requires cell cycle progression, IFN-γ production can occur prior to proliferation (2). We observed IFN-γ production in MLC with IFN-γ -/- responders and wild-type stimulators at approximately 30% of levels observed in MLC with wild-type responders and stimulators. This associated with a 20-30% increase in lytic activity over IFN-γ sufficient MLC, compared to the 100% increase observed in MLC with IFN-γR1 -/- responders. Others have reported similar results to ours with respect to increased lytic activity generated when IFN-γ is absent, however, lytic activity was not as increased as in our studies and the mechanism of action for this effect was not studied (3). The small differences in lytic activity may have been the reason why the investigators of these previous studies underestimated this aspect of the phenotype of IFN-γ deficiency. Our studies showed that the phenotype presented in those studies was masked by contaminating endogenous IFN-γ in their systems (stimulator cells). Since IFN-γR1 -/- responders are not affected by endogenous or exogenous IFN-γ, our initial studies reveal the phenotype of the complete absence of IFN-γ effects. Furthermore, by developing a truly IFN-γ deficient MLC using IFN-γ -/- stimulators and responders, we were able to bypass the problems of endogenous IFN-γ in the system. Production of IFN-γ by stimulator cells in MLC has also been a concern in human ELISPOT assays used to assess the amount of cross-reactivity of a host to a potential donor (4). Thus, although the effects of IFN-γ on the generation of lytic activity were hinted at in previous studies,

the actual difference between wild-type and IFN-γ deficiency was not appreciated until our studies presented here.

Regulation of IL-2 by IFN-γ, a major effector cytokine, constitutes an effective regulatory mechanism in effector T cell generation. Control of effector T cell generation by this negative feedback loop appears to be a two step process inherently linked. When CD4 T cells are activated in SLOs by DCs, they will produce IL-2 (5). As CD4 T cells proliferate, the collective amount of IL-2 produced increases and drives T cell expansion and effector differentiation. Differentiation to effector cells increases production of IFN-γ by CD4 and CD8 T cells (6). The strong anti-proliferative effects of IFN-γ help to limit CD4 T cell proliferation. By limiting CD4 T cell proliferation, the amount of IL-2 and help that can be provided for CTL generation is also limited (Figure 7a). Thus, the negative regulation of CTL generation by IFN-γ is largely via control of IL-2. Increased IL-2 production is also observed in cells lacking T-bet (7). Although we did not study the mechanism of IL-2 regulation by T-bet, we suggest that it is due to the decreased ability of T-bet -/- T cells to express IFN-γ (8), and thus decreased feedback on CD4 T cells. Alternatively, there may be a direct anti-proliferative effect by T-bet itself.

Few cytokines are as potent as IL-2 in enhancing CTL generation. The idea of IL-2 as a central T cell growth factor is based on its ability to maintain T cell clone viability, drive proliferation, and generate lytic activity in MLC (9;10). In support of this, MLC with IL-2 -/- responders manifest markedly decreased proliferation, and fail to generate any lytic activity (11). *In vitro* studies probably overestimated the role of IL-2 in CTL generation compared to its role *in vivo*. IL-2 -/- mice are able to fight off viral infections with similar kinetics as wild-type mice and do generate virus-specific CTL

responses (11). One must carefully analyze that initial study in IL-2 -/- mice: despite viral infections being equally eliminated in wild-type and IL-2 -/- mice, virus-specific CTL generated in IL-2 -/- mice are reduced three-fold. This suggests that although IL-2 is not necessary for *in vivo* CTL generation, it amplifies the CTL response. *In vivo* studies can at times be difficult to interpret given the redundancy that is often built into the immune system to ensure successful removal of a pathogen. It will be interesting to examine the phenotype of IL-2/IFN-γ -/- mice in studies similar to those performed in this project to determine the extent to which IL-2 drives CD4 and CD8 T cell generation, independent of the IFN-γ-IL-2 negative feedback loop.

7.3 EFFECTS OF IFN-γ ON CD4 AND CD8 T CELLS

IFN-γ effects on CD4 T cells have been studied more thoroughly than its effects on CD8 T cells. The focus on IFN-γ effects on CD4 T cells stems from their ability to differentiate into Th1 or Th2 phenotypes and differentially direct immunity. The CD8 field has only recently made considerable progress in understanding the responses of these cells to IFN-γ. Evidence currently suggests that IFN-γ has different effects on CD4 and CD8 T cells.

IFN-γ effects on CD4 T cell AICD. Many aspects of CD4 T cell biology are regulated by IFN-γ. Although CD4 T cells decrease IFN-γR2 surface expression and lose responsiveness to IFN-γ following repeated stimulation (12), naïve and short-term

activated CD4 T cells respond to IFN-γ. This means that IFN-γ likely regulates CD4 T cell function during most immune responses with a few exceptions in cases of chronic infections. One of the effects of IFN-γ is regulation of CD4 T cell homeostasis by promoting Fas-mediated AICD (13). Various effects on caspase expression have also been reported including increases in caspase 1, 11, and most important for its involvement in AICD; caspase 8 (13;14). However, the effects of IFN-γ are not unrestricted, since IFN-γ -/- mice do not suffer from lymphoproliferative disorders even in old age.

Immediately following activation, CD4 T cells produce IL-2 and are primed for Fas-mediated AICD. As the response continues, IFN-γ produced by effector T cells would enhance AICD by promoting caspase 8 expression. It is likely that IFN-γ is not necessary but does enhance the effects initiated by IL-2, which would explain why the lymphoproliferative disorder is observed in IL-2 -/- mice but not in IFN-γ -/- mice.

Anti-proliferative effects of IFN-γ/IRF-1 - basis for increased IL-2 production. IFN-γ is a more potent inhibitor of cell growth than other IFNs. These anti-proliferative effects are primarily mediated through IRF-1 (15). We have shown that MLC with IRF-1 -/- responders produce increased amounts of IL-2 similar to what we observed with IFN-γR1 -/- responders or IFN-γ deficient MLC. Since CD4 T cells are the main source of IL-2 in MLC we can deduce that IRF-1 deficiency in CD4 T cells leads to increased IL-2 levels as a result of increased proliferation. Thus CD4 T cell proliferation is tightly regulated, in part by IFN-γ, through IRF-1. Induction of the CDK inhibitors p21 and p27 and repression of CDK2 are critical in the ability of IFN-γ to arrest the cell cycle (16). Both

effects are mediated through IRF1 (15;17). Therefore it is not surprising that we found similar effects on IL-2 regulation in MLC when IFN-γ effects are absent and in MLC with IRF-1 responders. This brings about the question: if IRF-1 mediates cell cycle arrest in CD4 T cells, and many other cell types, what is it doing in CD8 T cells? If cell cycle arrest is also the principal effect of IRF-1 in CD8 T cells, then MLC with IRF-1 -/- responders should have generated increased lytic activity, as was observed in IFN-γ deficient MLC. Instead, IRF-1 -/- CTL generated in MLC are unable to lyse allogeneic targets, despite increased CD25 expression, suggesting that IRF-1 plays very different roles in CD4 compared to CD8 T cells.

Effects of IFN-γ on CD4 versus CD8 T cells. Effects of IFN-γ on CD8 T cells may parallel those of CD4 T cells, but some differences would be expected, given the different roles that they play in adaptive immunity. Whereas CD4 T cell proliferation is decreased by IFN-γ, CD8 T cell proliferation may actually be increased in response to IFN-γ. Whitmire et. al. found that IFN-γR1 -/- CD8 T cells do not expand as well as wild-type CD8 T cells in a LCMV infection model (18). This finding does not necessarily contradict our conclusions. We postulate that the CTL limiting effect of IFN-γ is via an action on CD4 T cells, indirectly limiting CTL generation. Thus our model would apply to CD4-dependent immune responses which lack CTL generation when CD4 T cells are absent. However, CTL responses to LCMV can be generated in the absence of CD4 T cells (19). Thus the observations of Whitmire et. al. may be applicable to LCMV infection and perhaps other viral infections, but not generally applicable to all immune responses. The *in vivo* role of IFN-γ in viral infections has generated many variable

results suggesting that immune responses to individual viruses vary greatly between different viruses. Much of this may also be a result of the lack of MHC class I induction in the absence of IFN-γ, given that this is critical for the detection of virally infected cells by CTL.

However, the overall effect when IFN-γ is not available to either CD4 or CD8 T cells is increased CTL generation due to the enhanced expansion of CD4 T cells. The effect may be a result of two separate but not mutually exclusive mechanisms: 1) increased IL-2 levels due to increased number of CD4 T cells and 2) increased help for CD8 T cells from the increased number of CD4 T cells. One way by which increased CD4 T cell numbers could enhance CTL responses is by inducing IL-12 production from DCs (20). IL-12 can in turn provide a third signal that enhances CTL generation and clonal expansion (21). Our studies were not able to address the effect of increased help for CD8 T cells resulting from increased CD4 T cells generated in the absence of IFN-γ. Such studies would benefit from the use of IL-2-/- /IFN-γR1 -/- CD4 T cells.

CTL are very closely related to NK cells and IFN-γ is important for the generation of NK lytic activity (1), thus it should not be surprising that it may also directly promote CTL generation. NK cells are able to kill without help or further differentiation and thus do not require interactions with other cells (22). CD4 T cells are important in the generation of CTL lytic activity in MLC. Thus results gathered from studies on CTL generation in MLC cannot be said to reflect direct effects on CD8 T cells. Resting splenic NK cell activity was decreased in IFN-γ -/- mice compared to wild-type (1), suggesting a role for basal IFN-γ production in the maintenance of NK activity. In this case the effect on NK cells is direct, since there is no immunologic stimulus given to

these mice prior to testing NK activity. Further studies focusing on the effects of IFN-γ on purified CD8 T cells are required to determine the direct effect of IFN-γ on CTL function and whether this is linked to the phenotype of IRF-1 -/- CTL.

Responsiveness to IFN-γ during CTL generation. The ability of T cells to respond to IFN-γ varies during the generation of the immune response. CD4 T cells regulate expression of IFN-γR2 and thus their ability to respond to IFN-γ, despite the maintenance of IFN-γR1 on the surface. Likewise, i*n vitro* polarized Th1 (CD4) cell lines are unresponsive to IFN-γ, due to downregulation of surface expression of IFN-γR2 (23). This occurs in a ligand-dependent manner since polarized Th2 cells normally express IFN-γR2 on the surface but downregulate expression when cultured with IFN-γ (12). Allospecific CTL clones are also unresponsive to IFN-γ due to decreased expression of IFN-γR2 on their surface (24). Prolonged expression of IFN-γR2 on CD8 T cells produces CTL that are activated, proliferate and produce cytokines normally but these cells are unable to lyse allogeneic targets (24). During primary immune responses CD8 T cells are initially responsive to IFN-γ but begin losing expression of both components of the IFN-γ receptor during the expansion phase and become unresponsive to IFN-γ (25). IFN-γ responsiveness is regained by the small number of cells that become memory cells. Such evasion of IFN-γ responsiveness could allow expanding CD8 T cells to escape the apoptotic and antiproliferative effects of IFN-γ. We and others (26), have shown that CD8 T cell apoptosis is not affected by IFN-γ, at least after allogeneic stimulation. Avoiding the anti-proliferative effects of IFN-γ would have obvious advantages during

the expansion phase of CD8 T cells, and may in part explain the high proliferation observed during this phase.

The role of IRF-1 during CTL generation. IRF-1 may play a critical role in proper development of CD8 T cells beyond the effects mediated by IFN-γ. Our results with IRF-1 -/- responders in MLC illustrate distinct differences between IRF-1 effects on CD4 versus CD8 T cells: proliferation and IL-2 levels are increased in MLCs yet lytic activity is not generated. The effect of IRF-1 on CTL generation is greater than that observed with the absence of IFN-γ effects suggesting two things: 1) IRF-1 expression is essential for the generation of lytic activity and 2) if the previous is correct then a degree of IRF-1 expression is not IFN-γ-dependent since IFN-γ -/- mice can generate properly functioning CTL. The vital role of IRF-1 for generation of fully functional CTL may be linked to the inherent decrease in the CD8+ T cell population as well as the thymocyte developmental defect (27). Further experiments are needed to elucidate the pathways affected by the lack of IRF-1 that lead to noncytolytic CTL. These experiments should include the use of CD8 T cells from TAP1 -/- or MHC class I -/- mice which, like IRF-1 -/- mice, have a decreased but present population of CD8 T cells (28;29). TAP1 -/- or MHC class I -/- CD8 T cells will determine how much of the phenotype observed is developmentally linked to decreased MHC class I expression in IRF-1 -/- mice.

7.4 *IN VIVO* EFFECTS OF IFN-γ ON CTL GENERATION

In vivo immune responses occur in conjunction with inflammation which in turn encompasses a complex milieu of numerous cytokines and chemokines. With our studies involving indirect regulation of IL-2 by IFN-γ, the question is complicated by the different number of cells which can produce one cytokine or the other, and by the importance of one cytokine in the context of many. *In vivo* studies must also take into account cell trafficking out of SLOs and into the inflamed tissue if antigen removal is to be used to determine the effectiveness of the response. It is also possible that certain cell types play critical roles *in vivo* and these may not be observed *in vitro*. Thus, due to the complexity of *in vivo* immune responses observations made *in vitro* may not always be observed *in vivo*.

IL-2 in in vivo immune responses. Although IL-2 is important for T cell responses *in vitro*, the requirement for IL-2 during *in vivo* immune responses remains poorly defined. The phenotype of IL-2 -/- mice was surprising, given the crucial role of IL-2 for *in vitro* T cell differentiation and proliferation (9). It is possible that in the absence of IL-2, compensatory functions of other cytokines sustain a basal level of T cell expansion and CTL generation. The most likely candidates to compensate are IL-15, IL-21 and IL-4, all of which can regulate CTL function, at least *in vitro* (30-32).

The apparent lack of *in vitro* T cell responses in the absence of IL-2 may underestimate the ability of IL-15 to function as a potent T cell growth factor *in vivo* simply because its effects may not be evident *in vitro*. IL-15 is abundantly produced *in vivo* in many different cells and tissues including APCs, and may serve as a growth factor to T cells in the periphery (33). Previous studies in our lab have shown only minimal

transcriptional regulation of IL-15 steady state mRNA during the rejection of kidney allografts regardless of the presence or absence of IFN-γ. Furthermore, a mouse IL-15 ELISA assay was not available until recently, hence we have not fully determined whether IL-15 levels are affected by the absence of IFN-γ in MLC, although it does seem unlikely. Moreover, we are interested in the effects of IFN-γ deficiency and not IL-2 deficiency. Since Th2 cytokines are often increased in immune responses in IFN-γ -/- mice (34), it could be worthy to determine whether IL-4 also contributes to the enhanced CTL generation in the absence of IFN-γ.

IL-2 can enhance CTL responses *in vivo* and has been used experimentally to enhance CTL responses to viral and tumor antigens (35;36). However, IL-2 can also have negative effects on T cell generation including induction of AICD (37). The positive or negative effects on CTL generation may depend on the stage of differentiation of the CTL. In LCMV-infected mice, IL-2 administration given during the expansion phase decreased the survival of the effector cells generated (38). However, IL-2 administration during the contraction phase increased proliferation and survival of virus-specific T cells. Unfortunately, we are unable to determine from that study what the role of endogenous IL-2 is on the immune response, or what effect IL-2 neutralization at different stages of differentiation would have. It would be useful to examine the effects of increased IL-2 production in the absence of IFN-γ on the generation of memory CTL. To understand this regulation *in vivo*, the kinetics of the increased IL-2 production caused by the absence of IFN-γ need to be examined.

Another important role for IL-2 is its contribution to the maintenance of self-tolerance and prevention of autoimmunity. This aspect of IL-2 is highlighted by the

development of lethal lymphoproliferative disorder accompanied with severe autoimmunity in IL-2 -/- mice (39). IL-2 is a growth factor for CD4 and CD8 T cells, including the recently discovered CD4+25+ regulatory T cells. IL-2Rβ -/- mice have decreased numbers of regulatory T cells (40), which correlates with autoimmunity. Neutralization of IL-2 also reduces natural regulatory T cell numbers, and elicits T cell–mediated autoimmune disease in normal mice (41). Our studies involving IL-2 neutralization did not seem to involve regulatory T cells, since the outcome was decreased CTL generation in IFN-γR1 -/- mice.

It is possible that the differences in the IL-2 neutralization protocols may account for the reason that we did not observe the generation of autoimmune disease. Setoguchi et. al. (41) elicited autoimmunity by neutralizing IL-2 but their protocol was different from ours. Our IL-2 neutralization protocol used a single dose of 250 μg per mouse compared to 1 mg, given twice, ten days apart. The anti-IL-2 mAb clones used also differed between the studies, as did the amount of time elapsed following IL-2 neutralization before the observations were made; 6 days in our studies versus 40 days by Setoguchi. The induction of autoimmune disease by IL-2 neutralization has also only been observed in BALB/c and NOD mice, while our studies were performed in C57BL/6 mice. BALB/c mice may be more predisposed to these autoimmune diseases and hence small changes in regulatory T cell numbers could readily produce autoimmunity. NOD mice already have a breakdown in tolerance mechanisms: they spontaneously develop a type 1 diabetes-like disease. Thus, studies involving CD4+25+ regulatory T cells in NOD mice can only serve a limited purpose. It is also important to note that IL-2 neutralization studies leading to autoimmunity have been performed in normal,

unstimulated mice and not in mice undergoing immune responses. The data is compatible with the conclusion that basal levels of IL-2 serve to maintain the regulatory T cell population, but increased levels of IL-2 during immune responses enhance the clonal expansion of effector T cells.

Possible role for IL-2 related cytokines in IFN-γ feedback. Similarities exist between IL-15, IL-21, and IL-2 in their actions on CD8 T cells and their abilities to be affected by IFN-γ. The most closely related cytokine to IL-2 is IL-21. Gene structure and proximity between IL-2 and IL-21 suggests that IL-21 arose as a result of gene duplication (42). Evidence also exists for a strong role for IL-15 in CD8 T cell homeostasis and expansion (43;44).

Both IL-15 and IL-21 could be affected by IFN-γ feedback although by different mechanisms. IL-15 is induced in monocytes by activation with LPS and IFN-γ (45). Whether IL-15 can be expressed in alternatively activated macrophages has not been examined. However, given the increased number of macrophages present in inflamed sites when IFN-γ effects are absent, it is possible that IL-15 levels may be increased in inflamed sites as a result. Although IL-15 transcript assessment results in our lab do not support this hypothesis we must remember that IL-15 regulation in APCs is complex. IL-15 is primarily controlled post-transcriptionally at the levels of translation and intracellular trafficking (30), hence changes may not always be evident in transcript levels.

IL-21 is the newest member of the γ_c chain family of cytokines. IL-21 displays superior ability to stimulate clonal expansion, differentiation, and survival of tumor-

specific CD8 T cells compared to IL-2 or IL-15 (32). Furthermore, unlike IL-15, IL-21 is produced by CD4 T cells and its receptor is expressed on T cells (42). Thus, IL-21 should also be affected by the anti-proliferative effects of IFN-γ in a manner very similar to IL-2. However, given that IL-21 is a newly discovered cytokine, reagents are not yet available for investigation.

7.5 IFN-γ AND THE EFFECTOR MECHANISMS OF ALLOGRAFT REJECTION

"Inflammatory" and "Generator" compartments in in vivo immune responses. Immune responses are initiated in SLOs leading to the generation of effector cells that home to the affected site. Thus we can split up the immune response into two separate compartments: 1) the "generator", composed of SLOs where effector cells are generated, and 2) the "inflammatory" compartment which is the site where the antigen that drives the immune response is found. In some chronic processes the inflammatory compartment may develop some SLO-like features such as germinal centers (46). In transplantation, the generator may be any of the SLOs depending on the location of the transplanted organ, while the inflammatory compartment is the allograft itself. It is likely that each of these compartments preserves its own set of strategies for maintaining immune cell homeostasis.

IFN-γ affects both the generator and inflammatory compartments. The effects on the generator compartment are the primary focus of these studies where we have

demonstrated that IFN-γ negatively regulates CTL generation by acting on CD4 T cells to decrease overall IL-2 production. However, due to the various effects that IFN-γ has on chemokine and adhesion molecule expression (47), the effects observed in the generator may not always be evident in the inflammatory compartment. In other words, the generator may not be the rate limiting step in the number of CTL entering the inflammatory compartment.

Four key chemokines believed to be responsible for the recruitment of activated T cells are regulated by IFN-γ. CXCL9-11 (Mig, IP-10, and I-TAC) and CCL5 all require IFN-γ for optimal expression (48). Activated T cells express CXCR3 and CCR5, thus the induction of CXCL9-11 and CCL5 by IFN-γ contributes to the proper recruitment of activated T cells to an inflamed site (49). Expression of CXCL9-11 may also decrease the recruitment of eosinophils by serving as an antagonist to the eotaxin receptor CCR3 (50).

CXCR3 expression is part of the effector T cell differentiation program similar to the increased expression of CD44. All immune responses are associated with increased chemokine expression by immune as well as non-immune cells and this is often accompanied with local IFN-γ production. CXCL9-11 and CXCR3 are important in mediating the recruitment of activated T cells and NK cells to the affected site. It was interesting to see increased CXCR3 expression in CTL generated in IFN-γR1 -/- mice in response to allogeneic tumor cell challenge, since expression of CXCL10 and 11 is increased by IFN-γ and CXCL9 requires IFN-γ (51). Would the increased number of CXCR3+ CTL in IFN-γR1 -/- mice be recruited to the affected site? The presence of CXCR3+CD8+ T cells in the peritoneal cavity of wild-type and IFN-γR1 -/- mice

following intraperitoneal challenge demonstrated that recruitment of CXCR3+ T cells was not reduced in IFN-γR1 -/- mice. Other studies in the lab have examined expression of chemokines in IFN-γ -/- kidneys rejecting in IFN-γ -/- hosts and found increased CXCL10 and 11 despite the absence of IFN-γ. Thus it is possible that IFN-γ-independent induction of CXCL10 and 11 mediates the recruitment of CXCR3+ T cells in IFN-γR1 -/- mice rejecting a peritoneal tumor. The cause of the recruitment of CXCR3+ T cells to an inflamed site in mice lacking IFN-γ effects would require in depth analysis of the activated T cell-attracting chemokines which are induced in the absence of IFN-γ. This result does however highlight the high degree of complexity that is built in to the chemokine system which ensures that the required cells will find their way to the site of infection.

Effector mechanisms in the presence and absence of IFN-γ effects. The effector mechanisms against allogeneic cells are altered by the presence or absence of IFN-γ. The larger number of allogeneic tumor cells remaining in the peritoneum of IFN-γR1 -/- mice compared to wild-type mice, despite equal CTL infiltration (Figure 6c – ii), suggests that the lytic mechanisms of CTL effectors are not directly responsible for destroying allogeneic cells. This is established in other allogeneic tumor models (52). If CTL are recruited to the site but clearance of the allogeneic tumor is hindered in IFN-γR1 -/- mice then: what is the effector function of CTL that clear allogeneic tumor cells?

CTL generated during allograft rejection compose a dominant portion of the lymphocytic infiltrate in the graft yet it is unclear whether rejection requires CTL-mediated lysis. Rejection of allogeneic tumors resistant to Fas- and TNF-mediated lysis

occurs at an equal rate in perforin -/- mice as it does in wild-type mice (52). Also, studies in our lab have shown that neither perforin nor granzymes A and B are required for the development of the T cell-mediated lesions in mouse kidney transplants which are diagnostic of rejection, namely tubulitis (53). Rejection of allogeneic mouse heart transplants is not impaired in perforin -/- mice despite severely reduced CTL lytic activity (54). It is possible that when granule mediated cytotoxicity is impaired, CTL rely on Fas-mediated lysis by expressing FasL on their surface to destroy target cells. Although FasL expression is increased in rejection (54;55), likely a result of the T cell infiltrate, FasL is not required for rejection of murine heart transplants (56). Altogether, these studies indicate that CTL lytic mechanisms are not required for allograft rejection which brings about the question: What is the effector cell and mechanism of allograft rejection? One possibility is that classically activated macrophages, in a delayed-type hypersensitivity reaction, are the key effector cells in allograft rejection.

CTL generation may also be affected by IFN-γ through the induction of NOS2 and NO production. The exogenous addition of NO via sodium nitroprusside to a primary human MLC inhibits both proliferation and generation of cytotoxic responses (57). Furthermore, NO also decreased IL-2 production and CD25 expression in these MLC and thus may play a role in our proposed negative feedback loop although we did not evaluate NOS2 or NO levels in our cultures.

Classical and Alternative activation of macrophages in alloimmune responses. Macrophages can undergo classical activation mediated by IFN-γ or alternative activation when exposed to IL-4 or IL-13. Classical and alternative activation are two ends of the

polarization spectrum for macrophages. One of the main differences between these is the decreased ability of alternatively activated macrophages for microbicidal activity despite their increased ability for phagocytosis (58). This is a result of the increased expression of arginase-1 in alternatively activated macrophages competing for L-arginine with NOS-2 whose expression is enhanced in classically activated macrophages (59). Many of the key Th1 associated effector functions of macrophages are decreased by their alternative activation including: production of proinflammatory cytokines including TNF and IL-12, and expression of high affinity Fcγ receptors (58). All this information suggests that classically activated macrophages are potent effector cells with the ability to kill microorganisms and possibly eukaryotic cells while alternatively activated macrophages are involved in allergic responses and tissue remodeling.

Classically activated macrophages are also abundantly found in rejecting allografts. Numerous cytotoxic mediators expressed by classically activated macrophages can injure allografts including TNF and NO. The interstitial infiltrate of rejecting human renal allografts can be as high as 60% macrophages (60). In allogeneic rat kidney transplants, over 70% of the intravascular leukocytes are monocytes (61). Furthermore, macrophages isolated from rejecting rat allografts exhibited increased cytotoxicity compared to those from untreated or isogeneic transplants (61). Depletion of macrophages by neutralizing the M-CSF receptor reduces interstitial macrophage accumulation and the severity of rejection (62). The concept that allograft damage during rejection occurs as a result of a nonspecific, delayed-type hypersensitivity reaction involving macrophages was first suggested nearly 50 years ago (63). Although the exact

mechanism of cytotoxicity by macrophages remains unclear, it is possible that it is enhanced by IFN-γ.

Rejection of allogeneic tumors and vascularized transplants may be mediated or at least enhanced by IFN-γ-activated macrophages. This may contribute to the reason why the allogeneic tumor cells were not as efficiently destroyed in IFN-γR1 -/- mice as they were in wild-type mice in our experiments. We can rule out the effects of IFN-γ on the tumor itself since IFN-γR -/- mice produce IFN-γ levels similar to those of wild-type mice in response to P815 challenge (Figure 6a). The IFN-γ-induced cytotoxic mechanisms in classically activated macrophages provide an effective way to destroy allogeneic targets. However, macrophages do not express receptors which would allow for the direct recognition of allogeneic cells. Thus a cross-talk must occur between graft infliltrating CTL and the nearby activated macrophages (64). In this cross-talk CTL would recognize allogeneic targets through their TCR and release effector molecules including IFN-γ. Effector molecules would act on nearby macrophages, which may or may not directly interact with CTL (64), inducing the release of macrophage cytotoxic mediators including TNF and NO. Macrophage derived cytotoxic mediators may work in conjunction with lytic mechanisms of CTL to destroy allogeneic targets and are likely capable of destroying targets even if CTL lytic mechanisms are absent.

Paradoxically, the lack of IFN-γ effects during alloimmune responses alters the immune response leading to increased numbers of macrophages in the inflammatory compartment. We found that the percent of macrophages in the peritoneum of IFN-γR1 -/- mice was more than three-fold higher than that found in wild-type mice. We did not examine whether the increased macrophage population represents increased recruitment

or increased *in situ* proliferation. The reason for the increased macrophage population in IFN-R1 -/- mice is unknown, but could be a result of the altered chemokine expression, the lack of anti-proliferative effects from IFN-γ, or a combination of both.

Thus it is possible that the classically activated macrophage is the principal effector cell in rejection of allogeneic tumor cells and maybe even allografts. This explains the increased number of P815 cells that remain in IFN-γR1 -/- mice compared to wild-type mice. It is not only important to have macrophages in the inflammatory compartment for destruction of allogeneic cells but the macrophages must be classically activated for adequate elimination of allogeneic cells. It is possible then that the effector function of CTL is the production of IFN-γ to classically activate macrophages in conjunction with CTL lytic activity.

Effects of IFN-γ in allograft rejection versus rejection of allogeneic tumor cells. IFN-γ must be received by the allograft in order to avoid accelerated ischemic necrosis. The cause of the ischemic necrosis is unknown, but based on recent results in our lab, is associated with the lack of MHC class I induction. But why is such damage observed in allograft rejection yet decreased allogeneic tumor clearance in IFN-γR1 -/- mice? Given the importance of the expression of MHC class I, we must take into account where donor and recipient MHC class I is expressed in comparing rejection of allografts to allogeneic tumors.

An important difference between vascularized organ rejection and rejection of intraperitoneal allogeneic tumor is the expression of donor versus host MHC on the vasculature. The vasculature where host leukocytes enter a vascularized allograft

expresses donor MHC. In contrast, leukocytes recruited to the peritoneum in response to allogeneic tumor challenge enter via host MHC-expressing vessels. It is possible that the lack of MHC expression on donor cells leads to ischemic necrosis but not lack of MHC expression on host cells. Furthermore, induction of MHC in the allograft requires IFN-γ whereas MHC expression in allogeneic tumors is generally quite high even in the absence of IFN-γ. Thus these two models are very different but both invoke a strong alloimmune response despite the differences in the inflammatory compartment. Effects of activating and inhibitory NK receptors on CTL may also play a role in mediating the necrosis of allogeneic tissue lacking MHC expression through an as of yet unknown mechanism.

Altered leukocytic infiltrate in the absence of IFN-γ effects. The increased granulocytes observed in IFN-γR1 -/- mice are in agreement with previous reports (48;65). However, the increased number of macrophages may be exclusive to alloimmune responses. Immune responses in mice lacking IFN-γ effects lead to increased accumulation of granulocytes including eosinophils and neutrophils, and this occurs in response to mycobacterial infections (66), autoimmunity in the central nervous system (65), and in alloimmune responses (67). The primary cause for the altered cell content of the leukocytic infiltrates in the absence of IFN-γ is altered chemokine expression (48;65), either through agonist or antagonist effects. IL-5, levels of which are increased in alloimmune responses in IFN-γ -/- mice, may also play a role in the eosinophil recruitment (67). Further exploration of the cause of the altered infiltrate and more importantly the effect of the individual cell types is necessary.

Homeostasis in the inflammatory compartment? The homeostatic mechanisms controlling T cells in the inflammatory compartment are not well understood. A critical concept governing T cell homeostasis is the competition for limited resources. Homeostatic equilibrium is achieved through cellular interactions and cytokines which serve to regulate survival and cell death (68). A possible explanation for the increased CTL generated in IFN-γR1 -/- mice to not be observed in the inflammatory compartment may be that the environment is only able to support a certain number of CTL or a certain number of leukocytes. We observed that the overall number of leukocytes is increased in the peritoneum of IFN-γR1 -/- mice rejecting an allogeneic tumor compared to wild-type mice. However, CTL numbers were no different in the inflammatory compartment of IFN-γR1 -/- mice compared to wild-type mice despite the increase in CTL effectors found in SLOs. If competition for CTL specific growth factor/cell interactions exists in the inflammatory compartment it would explain the lack of enhanced recruitment. But, if the competition is between leukocytes in general, then there may simply not be enough virtual space for the CTL in IFN-γR1 -/- mice as a result of the altered infiltrate which is observed early in the response. This second scenario is less likely given the cell specific competition observed for memory T cells (69). The search for whether such competition occurs in the inflammatory compartment during immune responses can involve studies using microarray technology to assess changes in the expression of thousands of genes, currently underway in our laboratory.

CTL generation is increased during alloimmune responses in the absence of IFN-γ. Whether this contributes to the rapid rejection of allografts in IFN-γ -/- mice is a

question that we have not been able to answer due to the complexity surrounding the effects of IFN-γ in this regard. By assessing alloimmune responses *in vitro* or in SLOs *in vivo* we were able to observe the increased generation of allospecific CTL in the absence of IFN-γ. It is unlikely that the accelerated rejection observed in IFN-γ -/- mice is due to the increased CTL generated. First, we must remember that, in the absence of IFN-γ, the increased CTL produced in the generator compartment is not reflected in the inflammatory compartment/allograft for reasons we do not currently understand. Second, the lack of IFN-γ has direct effects on the allograft, independent of its effects on the host immune system, which lead to the collapse of the microcirculation in these transplants (70). Whether the high expression of arginase 1, characteristic of alternative macrophage activation, is involved in the collapse of the microcirculation is the focus of current studies in the lab. It may prove useful to use selective deletion of IFN-γR1 on specific cell types to try to determine the effects of IFN-γ on an individual cell type in such a way that the problems with the complete absence of IFN-γ are avoided.

7.6 FUTURE DIRECTIONS

The experiments described herein define a novel mechanism for the regulation of CTL generation by IFN-γ in alloimmune responses. We now understand that regulation of CD4 T cell proliferation by IFN-γ helps to limit IL-2 production, and perhaps also the amount of help, for CTL generation. This is a previously unrealized pathway that indirectly controls the number of CTL produced both *in vitro* and *in vivo* by acting on

CD4 T cells. Experiments using mice where IFN-γR1 deficiency is restricted to CD4 T cells would be useful in examining the IFN-γ-IL-2 negative feedback loop in *in vivo* responses to immunologic stimuli outside of alloimmunity, including viral infections. Similar studies could also be done using adoptively transferred IFN-γR1 -/- CD4 T cells and wild-type CD4 T cells, either differentially labeled or expressing a marker different from those of the recipients (eg. Thy1.2). The advantage of both of these types of experiments is that the effects of IFN-γ are restricted to one cell type. Thus the problems with altered cellular infiltrate associated with the lack of systemic IFN-γ effects, would be avoided.

NOS2 expression is strongly induced by IFN-γ in macrophages (71). Since NO is capable of altering T cell function in a manner parallel to that of IFN-γ (57;72) it would be useful to examine the contribution of NOS2 and NO in our model. This could be done with the use of NOS2 -/- mice as well as NOS inhibitors both *in vitro* and *in vivo*.

Experiments of IRF-1 function in the generation of CTL lytic activity would benefit from microarray studies. Freshly isolated CD8 T cells, and activated CTL from IRF-1 -/- mice could be analyzed and compared to wild-type CD8 T cells and CTL. Microarray analysis provides an excellent tool for initial investigations searching for novel pathways regulating cell function. With the development of a core microarray facility in our lab, those experiments are now feasible. Furthermore, detailed studies examining T cell function in IRF-1 -/- CD4 T cells would provide further understanding of the IFN-γ-IL-2 feedback loop.

The studies of the effect of IFN-γ on cellular infiltrate would also benefit from the cell specific disruption of IFN-γR1. Such mice could be attained by the breeding of IFN-

γR1 -/- mice, generated using *Cre* technology, with transgenic mice expressing *lox* in a cell-type specific manner. These studies would determine the contribution of each cell type with respect to the altered cellular infiltrate in the absence of IFN-γ.

Our findings could be extended to a mouse kidney transplant model. Transplants could be carried out in IFN-γR1 -/- hosts or, using experiments suggested above, wild-type hosts where we are able to track wild-type and IFN-γR1 -/- CD4 T cells simultaneously. The parameters of CD4 T cell and CTL expansion could be examined along with characterization of the infiltrate in the allograft. These experiments will explore our findings in conditions that closely resemble the human transplant condition.

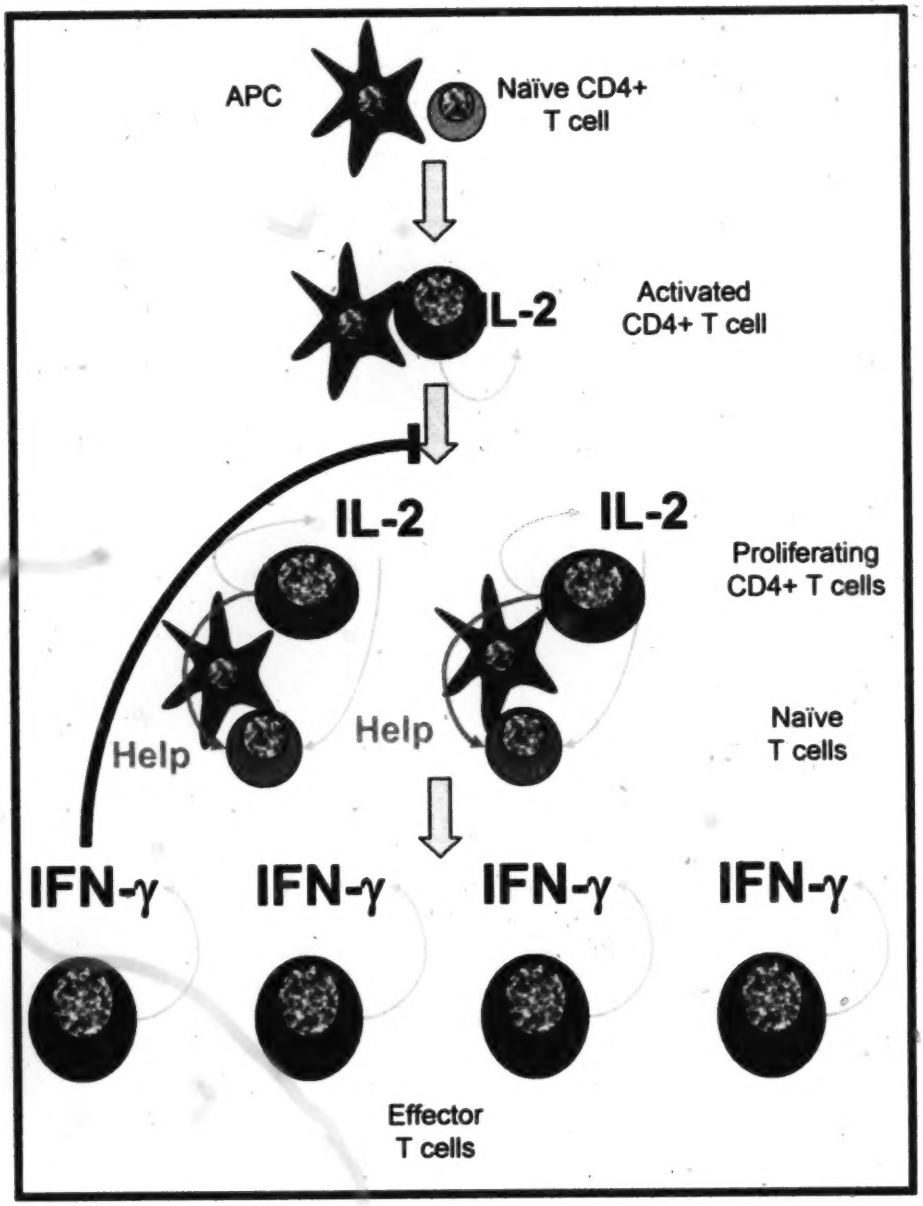
7.7 CONCLUSIONS

1. IFN-γ limits CD4 T cell proliferation, resulting in decreased IL-2 production *in vitro* and *in vivo*
2. Because IL-2 is necessary for optimal CTL generation, an IFN-γ-IL-2 negative feedback loop exists where IFN-γ limits CTL generation by decreasing IL-2 levels *in vitro* and *in vivo*
3. Effects of IFN-γ on CD4 T cell suppression of IL-2 are mediated through IRF-1
4. IRF-1 is required for the generation of cytolytic activity in CD8 T cells perhaps independent of its developmental effects
5. The IL-2-dependent increase in CTL generation in SLOs is not associated with increased CTL in the inflammatory compartment, indicating independent control of CTL generation and infiltration

6. IFN-γ plays a role in the control of allogeneic tumors independent of CTL cytotoxicity

7.8 FIGURE

Figure 7 a: IFN-γ-IL-2 negative feedback loop. Initial activation of naïve CD4+ T cells by APCs leads to production of IL-2 and proliferation of CD4 T cells. As CD4 T cells proliferate, the overall amount of IL-2 produced increases. IL-2 produced by proliferating CD4 T cells promotes the differentiation of effector T cells. Effector T cells in turn produce IFN-γ in response to antigen stimulation. IFN-γ produced by effector T cells acts on CD4 T cells to decrease their proliferation. As CD4 T cell proliferation is decreased, the overall production of IL-2 is also decreased leading to reduced effector T cell generation.


APC
Naïve CD4+
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Activated
CD4+ T cell
IL-2
IL-2
Proliferating
CD4+ T cells
Help
Help
Naïve
T cells
IFN-γ
IFN-γ
IFN-γ
IFN-γ
Effector
T cells

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